THE EFFECTS OF HARVESTING PROCEDURES ON PHYSIOLOGICAL AND BIOCHEMICAL PROPERTIES OF CHINOOK SALMON (*Oncorhynchus tshawytscha*) WHITE MUSCLE PRIOR TO AND DURING FROZEN STORAGE

A thesis submitted in partial fulfilment of the

requirements for the Degree of

Master of Science in Zoology

in the University of Canterbury

University of Canterbury

2008

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List of Abbreviations

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
CF	Condition Factor
CPK	Creatine phosphokinase
CW	Chilled water
FAD	Flavin adenine dinucleotide
FFA	Free Fatty acid
GSI	Gonadosomatic index
GSH	Glutathione
HCSS	High concentration salt solution
HK	Hexakinase
HSI	Hepatosomatic index
Hx	Hypoxanthine
IMP	Inosine monophosphate
Ino	Inosine
MDA	Malondialdehyde
NAD^+	Nicotinamide-adenine dinucleotide
PCA	Perchloric Acid
PCr	Phosphocreatine
PM	Post mortem
RM	Red Muscle
ROS	Reactive Oxygen species
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCA	Tricarboxylic acid cycle
WM	White Muscle

Abstract

The object of this thesis was to investigate the role of two different harvest protocols on the post mortem physiology of Chinook salmon, and associated deteriorative processes that occur during frozen storage of the white muscle tissue. The two different harvest methods employed, termed 'rested' and 'exercised', were selected because of the contrasting levels of activity of the animal prior to, and upon, slaughter. While the latter represents conventional harvest techniques

Rested and exercised harvesting protocols produced tissue in significantly different physiological states. Immediately post harvest, rested tissue maintained high metabolic energy stores of ATP and glycogen within the tissue, with low concentrations of tissue and plasma lactate. Exercised tissue exhibited near depleted concentrations of ATP and glycogen and a marked metabolic acidosis and lactate accumulation. When frozen immediately post harvest, rested white muscle tissue stored at -19°C showed no significant changes in these metabolite concentrations over a six month period of profiling. However, during storage of rested tissue at -9°C, hydrolysis of ATP and glycogen with no coincident increase in lactate was observed. No significant changes in metabolite levels were observed within exercised tissue stored at -19 and -9°C, owing to the lack of metabolic energy stores. Transfer of tissue from frozen (-80 and -19°C) to chilled (-1 and +4°C) temperatures witnessed a rapid depletion of tissue ATP and glycogen stores, with rapid increases in tissue lactate concentrations. This metabolic activity was more significant in rested tissue owing to the larger concentrations of metabolic energy stores. This metabolic activity was identified to occur between the temperatures of -3 and -1.5°C and occurred abruptly (i.e. ATP concentrations depleting in less than one hour) in time.

During frozen storage (-19°C and -9°C), harvest treatment had no significant effect on lipid oxidation processes. However, rested tissue showed a significant ability to retard lipid oxidation processes once removed from frozen storage and placed at chilled

temperatures. Throughout six months storage at -19°C storage, harvest treatment had a significant effect on the rate of protein denaturation as rested tissue consistently held higher concentrations of soluble protein over the storage period. No significant effect was observed between treatments in the rate of protein denaturation during one month frozen (-19°C) then chilled (+4°C) storage.

In a supplementary frozen (-80°) then chilled (-1°C) storage experiment, post mortem storage of rested, whole fish, at chilled (+5°C) temperatures prior to white muscle excision and freezing, was compared to rested and exercised tissue in which the white muscle had been excised and then frozen immediately post harvest. In this experiment rested tissue exposed to a 6 or 24 hour post mortem chilled storage period demonstrated significant retardation of lipid oxidation processes when compared to rested white muscle tissue that was excised and frozen immediately post harvest. Further comparison of the six and 24 hour post mortem stored tissue showed a significant increase in lipid oxidation products after 21 and 24 days chilled storage, respectively. Comparison of results from the six and 24 hour post mortem storage experiment were bordering on significance ($p=0.083$), warranting further investigation on the effect of post mortem storage of rested tissue on lipid oxidation processes.

CHAPTER 1

Introduction

General Introduction

The harvesting of fin-fish involves a transition of the musculoskeletal system of fish from a physiologically active, dynamic entity to a comparatively static food product that is valued for the nutritional aspects of its composition. This transition is not immediate in nature, but is instead dictated by the ability of the cells to continue metabolic activity and maintain cellular viability for a limited time period *post mortem.*

Viewing the harvest process as a progression from physiologically active muscle through to a food product, inherently suggests that the quality of the food product is at an optimum while in a state resembling that of the living tissue. However, the state of quality that exists when the animal is harvested or captured can neither be maintained, nor improved, as processes of degradation (e.g. *rigor mortis*, autolysis) begin immediately after death (Amalcher, E. 1951; Robb, D. 2002). Measures that slow spoilage and degradation processes can be employed but will only delay deterioration from the initial state of the product. For this reason, attaining a raw product in an optimal physiological state, *peri mortem*, is beneficial to providing a high quality food product.

In order to achieve fish in an optimal physiological state, care has to be taken to not induce high levels of activity or stress the fish upon capture or harvesting. Fish handled this way will show less physical damage, with minimal perturbation of physiological systems/metabolic processes. Coinciding with careful harvest processes numerous quality benefits have been observed in freshly harvested fish, such as extended rigor periods, decreased rigor tensions, decreased gaping and elevated 'freshness' (Robb 2002). However, limited research has been conducted examining the effects of pre-slaughter stress and exercise on subsequent sub-zero storage of fish tissue.

This thesis reports on an investigation into sub-zero storage properties of salmon tissue in different physiological states as derived from different harvest methods. Thus, an

introduction into fish muscle and exercise physiology is presented followed by an introduction into 'quality' as it pertains to fish as a food product.

Teleost Skeletal Muscle – Structural and Functional Aspects

Skeletal muscle comprises 60% of the anatomical composition of teleost fish (Sanger, A.M. & Stoiber, W. 2001). This large volume of muscle is required for locomotion in the dense water environment. Physiologically, teleost muscle is of significance owing to the energetically expensive nature of its function (Willmer, P. et al. 2005). Commercially it is important as muscle tissue is generally favoured for its eating quality and nutritional benefits.

Muscle Appearance and Structure

Fish muscle falls into three major categories – cardiac, smooth and striated muscle. Striated type muscle, also known as skeletal muscle, is of primary importance to this thesis.

Teleost muscle tissue viewed after dissection from the mid sagittal plane is observed to have a dorsal-ventrally arranged W shape pattern. These W shaped blocks of tissue are the myotomes or blocks of muscle tissue. Collagenous connective tissue (myosepta) joins the myotomes together. Within the myotomes numerous parallel arranged muscle fibres can be seen on the macroscopic scale. Within the muscle fibres numerous subunits or myofibrils are found which contain the sarcomeres, the contracting elements of the muscle tissue. Each sarcomere unit is made up of two different types of protein filaments – actin and myosin. Associated with the myosin are troponin and tropomyosin complexes that assist in the contraction and relaxation of the muscle filaments (Willmer, P. *et al.* 2005).

Energy Production in Muscle

Skeletal muscle can divided into two major sub-types – red and white muscle. Red muscle (RM) is lower in abundance than white muscle (WM) and is seen as a thin strip along the major horizontal septum of most fish (Johnston, I.A. 2001). RM fibres are small in diameter (25-45μm) and specialised for long term, low intensity exercise (Sanger, A.M. & Stoiber, W. 2001). The muscle is highly vascularised (providing

respiratory gases, fuel substrates and waste removal functions), with high myoglobin concentrations (which give the muscle its coloration) and high volume densities of mitochondria (20-50%) (Johnston, I.A. 2001). White muscle consists of fast twitch fibres that are multiply innervated and possess much lower abundances of mitochondria, myoglobin and blood vessels (Willmer, P. *et al.* 2005).

Muscle function (contraction and relaxation) of both WM and RM relies on the activation of actomyosin (actin and myosin) cross-linking. This interaction requires ATPase activity and associated ATP hydrolysis to Adenosine diphosphate (ADP). However, endogenous stores within WM only consist of 5-8µmol.g^{-1}of ATP, with RM containing even lesser stores (Hochachka, P.W. 1985). To continually replenish ATP, ADP must be phosphorylated by one, or any combination of three major sources – high energy phosphagens, anaerobic metabolism and aerobic metabolism.

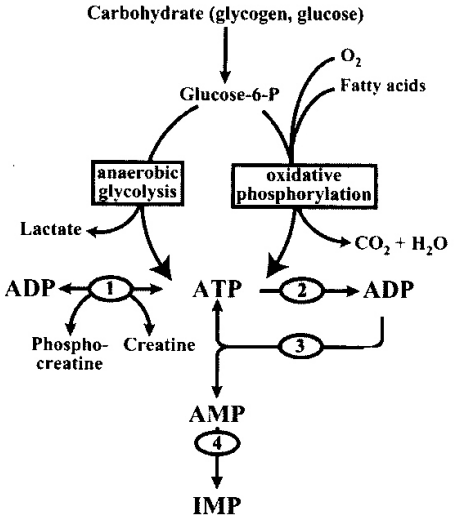

Figure 1- 1: Overview of Cellular Respiration.

Source: (Joanisse, D.R. 2004)

High Energy Phosphagens

High energy phosphagens (such as PCr) provide the most rapid mechanism by which hydrolysed ADP can be converted back to ATP. The primary phosphagen utilised for ATP replenishment amongst teleosts is creatine phosphate (PCr) which is stored as a total pool size of up to 30µmol.g^{-1} in the skeletal muscle, as well as the myocardium and the brain (Hochachka, P.W. 1985). PCr acts by donating its phosphate group to

ADP, assisted by the enzyme creatine phosphokinase (CPK) (see Figure 1-2). CPK activities are highest in WM, with animals capable of high burst activity showing higher enzyme activity (Hochachka, P.W. 1985). Due to the limited pool of PCr available, phosphagen based work can only be sustained for a very limited period before other sources of ATP are required to sustain metabolic processes.

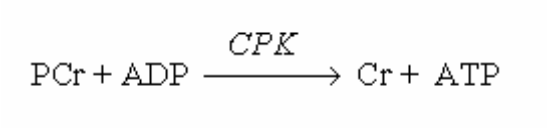

Figure 1- 2: Phosphorylation of ADP by PCr

Aerobic Metabolism

The basal metabolic needs of physiological systems are met by aerobic metabolism (Withers, P.C. 1992). Aerobic metabolism can generate ATP by the oxidation of carbohydrates, lipids and protein within both the cytosol and mitochondria. All oxidative metabolic processes involve the TCA (aka Krebs) cycle, and the associated oxidation of acetyl-CoA to CO_2 and H_20. Within the TCA cycle the proton acceptors Nicotinamide Adenine Dinucleotide (NAD^+) and flavin adenine dinucleotide (FAD) are converted to NADH and $FADH_2$, these compounds must be regenerated to allow metabolic processes to continue cycling. Regeneration of NAD^+ and FAD through the electron transportation chain, within the mitochondria, allows further generation of high energy phosphagens/ATP and in fact comprises the largest fraction of the total yield from the oxidation of the initial fuel substrate. In entirety, the oxidation of one molecular unit of glucose to CO_2 and H_20, via the TCA and electron transport chain, produces 36 molecular units of ATP (Withers, P.C. 1992). A diagrammatic representation of aerobic glycolysis is presented below (Figure 1-3).

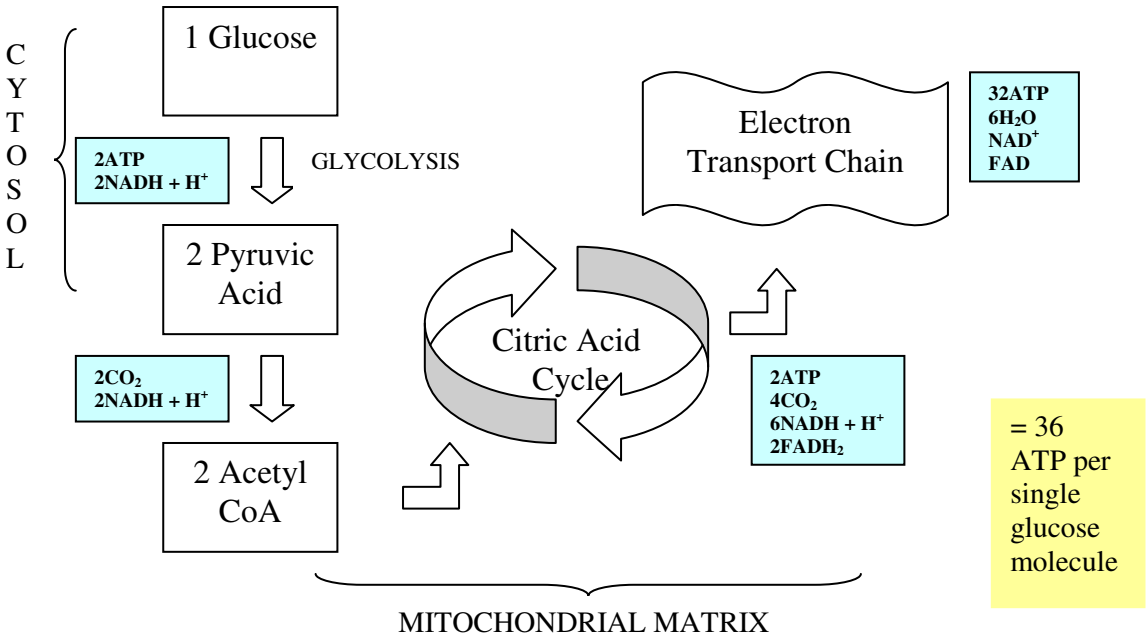

Figure 1- 3: Overview of Glycolytic Aerobic Respiration

Anaerobic Respiration

Anaerobic metabolism occurs under oxygen limiting conditions, i.e. when oxygen availability or perfusion is limited. Under anaerobic conditions the only fuel reserves available are carbohydrate stores. As in the earlier stages of aerobic metabolism, glucose is oxidised to pyruvate, with the production of two molar units of ATP and the generation of NADH and $FADH_2$. In order to regenerate NAD^+, pyruvate is converted to L-lactate, allowing anaerobic metabolic processes to continue for a limited time. Lactate as the by-product of anaerobic metabolism will typically be removed upon return to aerobic respiration. However, prolonged utilisation of anaerobic metabolic processes can have other effects on vertebrate (including teleost) physiology, as will be discussed later.

Anaerobic metabolism poses advantages as ATP is produced more rapidly than under aerobic conditions. However, the process is inherently less efficient, as under anaerobic conditions a net gain of two molar units of ATP is achieved from each molar unit of glucose.

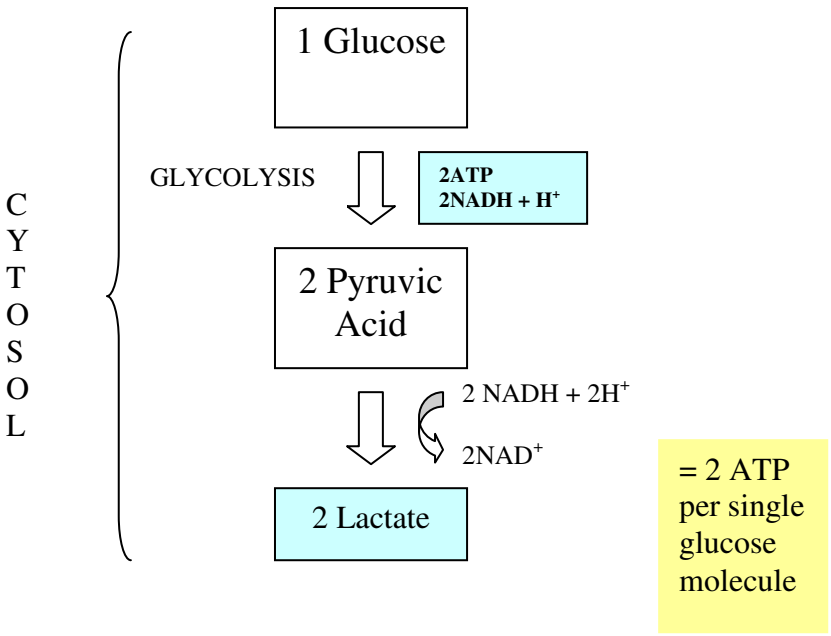

Figure 1- 4: Overview of Anaerobic Metabolic Pathways

Metabolic Energy Stores

Carbohydrates, lipids and proteins are the three main energy reserves able to fuel cellular metabolism (Withers, P.C. 1992). Carbohydrate metabolism predominantly takes the form of glycolysis, where glycogen (the stored form of glucose) is catabolised, releasing free glucose within the cytosol. Each molar unit of glucose is then oxidised to two molar units of pyruvate, producing two molar units of ATP. Pyruvate molecules can then readily be converted to Acetyl-CoA or lactate for aerobic or anaerobic metabolism, respectively.

Lipids, in the form of triglycerides, are a further fuel that is a major source of metabolic energy for movement (Withers, P.C. 1992; Tocher, D.R. 2003). Triglycerides, typically stored within the adipose tissue of vertebrates will, through the action of lipases, release glycerol and fatty acids. These substrates are then diffused into the blood for transport to the muscle tissue. Glycerol, a carbohydrate, can be metabolised in the same way as glucose, while the fatty acids portion can be metabolised through the β-oxidation chain to form many acetyl-CoA units which may then be incorporated into the TCA cycle.

Proteins, when hydrolysed to their constituent amino acids, can be metabolised to either pyruvate or Acetyl-CoA (Withers, P.C. 1992). Protein appears to be a minimally

utilised fuel substrate in teleost muscle (Moyes, C.D. et al. 1989; Kieffer, J.D. et al. 1998).

As exercising and ischemic skeletal muscle utilises glycolytic anaerobic metabolic processes (Wang, Y.X. et al. 1994; Black, S.E. et al. 2004), these pathways will feature within this thesis.

Teleost Skeletal Muscle – Dynamic Aspects relating to Whole Fish Physiology

Exercise Metabolism

Fish propulsion during low-intensity, long term activity, is driven primarily by the contractive forces of the RM. Metabolic fuel use by mitochondria within the RM appears to primarily involve pyruvate and fatty acid oxidation with small contributions from the amino acids (Moyes, C.D. *et al.* 1989). However, during aerobic exercise there is a general lack of agreement on the comparative rates of pyruvate and fatty acid utilisation, with acclimation and swimming speed all seeming to alter the rates of metabolic fuel usage (Kieffer et al., 1998)

Conversely, during high intensity, burst-type exercise WM fibres are recruited to sustain the high levels of activity. The general consensus is during burst type exercise cellular processes are driven by glycolytic anaerobic metabolism (Moyes, C.D. *et al.* 1989). Contractive activity in WM fibres, with their low perfusion rates and low concentrations of myoglobin and mitochondria, readily exceeds the aerobic capacity of the cells and thus must rely on anaerobic processes (Hochachka, P.W. 1985). Evidence of this anaerobic activity can be demonstrated in rainbow trout, where exhaustive exercise involves a 40% depletion of PCr and ATP levels, 90% depletion of muscle glycogen, and increases in plasma lactate, pyruvate, glucose, ammonia, and inorganic phosphate (Wang, Y.X. *et al.* 1994). Combined with the metabolic perturbations listed, acid-base perturbations of both a respiratory and metabolic origin also occur in the exercising and, or exercised fish. The primary reason for shifts in the acid base balance in an exercising fish is the net increase in H^+ production during ATP hydrolysis – a metabolic acidosis (Hochachka, P.W. 1985).

Recovery from exercise and the return to 'normal' physiological conditions is a homeostatic phenomenon, providing the exercise event was not so severe as to cause irreparable internal perturbations (which will prevent recovery and will lead to mortality) (Wood, C.M. et al. 1983). The period until recovery is complete varies between species, and the time course varies for different facets of the perturbation associated with the exercise event.

Hormonal Systems / Endocrinology

Exercise physiology as described above was presented in isolation from the endocrine system. However, under real situations the two systems are tightly associated. High intensity exercise is often associated with a fright or stress response. This activates endocrine responses including cortisol and the catchecholamines (which include adrenaline and noradrenaline), as well as lesser studied responses including glucagons, insulin, T3 and T4 (Milligan, C.L. 1996). Endocrine responses to stress provide an adaptive function that alters the physiological state of the animal, providing an enhanced ability to cope with stressors.

Both cortisol and catchecholamines alter numerous aspects of teleost physiology. The catchecholamines act upon the respiratory and cardiovascular system enhancing blood flow and oxygen diffusion/transport capabilities while also stimulating glycogenolysis within the teleost liver and elevating blood glucose levels (hyperglycemia) (Wendelaar Bonga, S.E. 1997). Cortisol acts to enhance ion transporting activity of the branchial structures as well as increasing circulating blood glucose levels via gluconeogenesis (Wendelaar Bonga, S.E. 1997).

The two different hormones possess different temporal behaviour. Catchecholamine release is immediate and short lived, with concentrations of adrenaline and noradrenaline peaking immediately prior to exercise before returning to normal values after 2 hours in rainbow trout (Milligan 1996). Conversely, peak values of plasma cortisol are reported in rainbow trout two hours after exercise and drop to normal values after six hours, although elevated levels can remain in chronically stressed animals (Milligan, C.L. 1996; Wendelaar Bonga, S.E. 1997). With reference to exercise physiology, endocrine up-regulation of glycolysis is of considerable importance.

Stress Physiology

'Stress' is a term widely used in the anthropogenic and aquaculture realms. The term can be defined in numerous ways but, for the purposes of this thesis, stress will be interpreted, in line with Wendelaar Bonga (1997), as a condition in which homeostasis is threatened or disturbed as a result of internal or external stimuli. The stress response is often viewed in general terms, owing to the common physiological response triggered by stressors of varying nature (Iwama, G.K. et al. 2006). This generalised response can be adaptive – where homeostasis is maintained in spite of the stressor, or maladaptive – where the stress response inhibits normal physiological functioning of the animal. The stress response can be separated into three responses: primary, secondary and tertiary. The primary response involves a neuroendocrine response that releases stress hormones, catchecholamines and cortisol (Iwama, G.K. *et al.* 2006). The secondary response involves the up and down regulation of physiological processes which allow the animal to cope with the stressor (i.e. the mobilization of energy substrates to fuel escape activity). The tertiary response involves whole animal and population scale responses to stress, also referred to as 'chronic stress' (Iwama, G.K. *et al.* 2006). This response is associated with impaired physiological function of the organism owing to the continual (lower level) activity of the integrated stress response.

Fish Physiology in the Aquaculture Setting

Growth of fish in intensive culture facilities has the potential to place excess physiological pressures on the stock. Factors such as oxygen concentrations, water quality and stocking densities all need to be managed so no undue physiological pressures are placed upon the fish. However, the intensive nature of aquaculture dictates that the fish are grown in an environment altered from their natural setting and are managed so as to achieve optimal growth. Day to day management procedures including crowding, handling, transportation and disease treatments, can have an immediate and longer term/compound effects upon the physiology of the fish (Schreck, C.B. & Lorz, H.W. 1978; Wedemeyer, G.A. 1996; Wendelaar Bonga, S.E. 1997; Yada, T. & Nakanishi, T. 2002).

Like all fish handling practices, the harvest procedure has the potential ability to alter the physiology of the fish from that of a rested state. Typically, a harvest will involve the marshalling and/ or crowding of fish to ready them for harvest. Numerous different

harvest techniques exist, many of which involve the fish suffering high density crowding (to increase manageability) which often results in typical escape behaviour owing to adverse water conditions or high fish densities. Even before the harvest procedure has begun, abrupt and forceful marshalling procedures may lead to the general stress response being elicited and the energy stores within the fish depleted. Also, physical injury due to high levels of activity can occur such as damage to the scales or epithelial layers, as well as tissue gaping, broken vertebrae and any other injury that may detract from the appearance and hence 'quality' of the fish (Robb, D. 2001).

During the harvesting procedure the fish are exposed to a lethal event, which may include: narcosis caused by prolonged exposure to air or deoxygenated water (CO_2 narcosis) inducing death by anoxia, application of an electrical current; percussive stunning (either manually or by an automated unit) inducing an irrecoverable concussion; or exsanguination (either in air or water) causing death by anoxia and/or ischemia. Of these examples, only the application of a percussive stun and an electrical current immediately render the fish insensible upon treatment (when applied correctly) and can be considered humane slaughter techniques (van de Vis, H. et al. 2003). However, these two methods can potentially cause physical injury to the fish. An incorrectly applied stunning action may cause bruising and eye damage. Application of an electrical current can cause carcass haemorrhages and vertebrae damage (Robb, D. 2001). Both of which will affect the visual appearance of the fish, resulting in downgrading of the final product.

Mitigating Adverse Physiological Responses during the Harvest

Rested Harvesting is a fish harvest method that incorporates the aquatic anaesthetic AQUI-S® and gentle marshalling procedures. AQUI-S™ is a food grade anaesthetic comprising isoeugenol as the active ingredient. AQUI-S™ is added to the water column at recommended doses of 17-20ppm (species and purpose dependent) and subsequently sedates then anaesthetises the fish over the exposure period. During introduction of the anaesthetic solution and subsequent induction into anaesthesia, no adverse responses are shown by the fish, providing exposure concentrations are less than 60ppm (Hill, J.V. & Forster, M.E. 2004). After approximately 30 minutes exposure to the recommended dose of AQUI-S™, the fish are insensitive to handling (i.e. strong

pressure on the caudal peduncle elicits no response). At this point the fish can be euthanized by ablation of the brain, percussive stunning or asphyxiation with minimal adverse response. Rested harvesting provides a method of harvest that limits escape behaviour and high levels of activity, suppressing the stress response and the utilisation of energy reserves (Iversen, M. et al. 2003; Bosworth, B.G. et al. 2007)

Teleost Skeletal Muscle – A Consumable Product

To provide a fresh, high quality product, harvested fish should be provided for sale to the consumer as soon as logistically possible. As time elapses following the harvest, numerous deteriorative processes can have an effect on the quality of the muscle tissue. Over time these processes culminate in a poor quality, less desirable, food product.

Quality Deterioration Pathways

The first observable process associated with quality deterioration and the conversion of skeletal muscle from a physiologically active system to a physiologically inactive system is the onset of *rigor mortis. Rigor mortis* is the phenomenon whereby actomyosin complexes bind, leaving the skeletal muscle in a contracted state and the fish rigid. The binding of actomyosin complexes occurs as the cells lose their ability to generate ATP, either through depletion of energy substrates, or cessation of enzymatic activity within the acidic environment (Robb, D. 2002). The "pre rigor period", that is the time period following slaughter but prior to the onset of *rigor mortis,* is determined by the levels of activity experienced prior to slaughter and the physiological state of the animal (e.g. metabolic acidosis, high energy phosphagen stores and glycogen reserves) (Robb, D. 2002). Fish handling and storage prior to and during the rigor event can have downstream effects on product quality. Rigor onset at high temperatures result in high rigor tensions which may cause tissue gaping - a separation of the myotomes at the connective tissue (Huss, H.H. 1995). Excessive handling or filleting of a carcass in rigor can have the same effect (Ando, M. et al. 1996). The resolution of *rigor mortis* occurs after 2-4 days in fish stored on ice (Erikson, U. 2001).

Although the mechanism behind the resolution of rigor is not well understood, the event itself correlates with autolytic changes in the muscle. The catabolism of the high energy phosphates, notably ATP, is the first notable product of autolysis. High energy

adenylates successively degrade to form Adenosine diphosphate (ADP), Adenosine monophosphate (AMP), Inosine monophosphate (IMP), Inosine (Ino) and hypoxanthine (Hx). Collectively these catabolites have been used to identify the K value of freshness, a ratio of Ino + Hx concentration to the summed concentration of ATP, ADP, AMP, IMP, Ino, Hx measured at one point of time. Rupture of the cellular organelles, either through physical handling or autolytic degradation, also accelerates the rate of quality deterioration of fish tissue. Proteases released from the lysosome and/or cytosol act to catabolise proteins forming free amino acids and peptides. These products are often related to the softening of the muscle tissue. Raised concentrations and activation of the notable proteolytes cathepsins and calpains are widely suggested to be related to the softening of teleost skeletal muscle. Cathepsin-L has been implicated in the softening of salmon tissue during spawning and post mortem degradation (Yamashita, M. & Konagaya, S. 1990; Aoki, T. & Ueno, R. 1997; Iwama, G.K. *et al.* 2006; Salem, M. et al. 2006). While the calpains are widely known to assist in tenderisation of mammalian meat, their influence on textural degradation of teleost muscle remains to be fully explored. (Cheret, R. et al. 2007).

The high lipid content of fatty fish (such as salmon) renders the fish susceptible to lipid oxidation, and associated rancidity development. Lipid oxidation, an autocatalytic reaction (meaning the reaction rate increases as the reaction proceeds), occurs predominantly within the polar (phospholipid) fraction of the fish tissue, as opposed to the neutral lipid fraction which include triacylglycerols (Igene, J.O. et al. 1980; Erickson, M.C. 1997). The lipid oxidation process occurs in initiation, propagation, chain branching and then termination steps. Initial steps of the reaction involve the presence of a free radical initiator that cleaves a proton from a saturated carbon-carbon bond. The resulting lipid radical then propagates through a reaction with oxygen to form a peroxy radical ($LOO^{\cdot}$), which will then either attack another lipid molecule or the starting lipid, removing a proton and forming a hydroperoxide (LOOH). Upon formation of the hydroperoxide the proton donor is itself transformed into a radical. These hydroperoxides are commonly referred to as the primary oxidation products. Branching of hydroperoxide(s) form two further radicals (either $RO^{\cdot} + OH^{\cdot}$ or $ROO^{\cdot} + RO^{\cdot}$), increasing the number of free radicals present in the system that can undergo propagation. This is the origin of the autocatalytic, or chain reaction. Decomposition of hydroperoxides form the secondary oxidation products. These consist of numerous

ketones and aldehydes. Further reaction of secondary oxidation products with amino acid products forms tertiary oxidation products. Within each of these initiation, propagation and chain branching steps, competing termination reactions occur where two radicals react to form a non-radical product.

Initiation:

$$RH + In^{\cdot} \rightarrow R^{\cdot} + InH$$

Propagation:

$$R^{\cdot} + O_2 \rightarrow RO_2^{\cdot}$$
$$RO_2^{\cdot} + RH \rightarrow ROOH + R^{\cdot}$$

Branching:

$$ROOH \rightarrow RO^{\cdot} + OH^{\cdot}$$
$$2ROOH \rightarrow ROO^{\cdot} + RO^{\cdot} + H_20$$

Termination:

$$2R^{\cdot} \rightarrow RR$$
$$R^{\cdot} + RO_2^{\cdot} \rightarrow ROOR$$

Where R = Fatty Acid
$In^{\cdot}$ = Initiating Radical
ROOH = Fatty acid hydroperoxide
$RO_2^{\cdot}$ = Peroxy Radical
$RO^{\cdot}$ = Alkoxy Radiacal

Figure 1- 5: Reaction Scheme Demonstrating the Auto-oxidation of Lipids

Newly harvested fish are sterile. This is because the immune system of the fish prevents bacteria from growing in the flesh (Ruskol in Huss, 1995). However upon death and throughout storage the growth of numerous micro-organisms will occur upon exposed surfaces of the fish. A wide range of different strains of bacteria can grow on the tissue, however not all of these will have a role in the spoilage process (i.e. formation of off odours and flavours) (Shewan, J.M. 1961).

Evaluation of Quality

'Quality' is a widely used term in the aquaculture context. It is used to attribute superior or enhanced characteristics of cultured or captured fish whether it is in relation to the rearing conditions, production methods, nutritional value and/or marketable presentation of the fish. For the following section the term 'quality' will represent both the physiological condition of the fish and the degree of tissue degradation.

Within both commercial and scientific settings, sensory methods have been adopted in order to grade fish in respect to quality. Such evaluation can be used for determining the product freshness, or alternatively can be used to rate the consumer appeal of the

product. Numerous measures including: taste and smell (e.g. freshness, off-flavours/smells); visual appearance (e.g. colour, gaping); and texture (e.g. firmness, elasticity), can be made on the whole fish or on fillet portions, with various criteria used to qualify and grade the product. To achieve fair sensory evaluation, numerous aspects of the sensory assessment need to be standardised. Additionally, the subjective nature of these tests requires that assessors are all trained and are able to give standardised responses to variable samples. Guidelines from the International Standards Organisation (ISO) are often the basis of sensory evaluation experiments.

Non-sensory evaluation often involves measurements of physical, chemical and biological properties of a harvested fish. The range of non-sensory analytical techniques that can be used to determine fish quality is exhaustive and can include measurements of bacterial counts (e.g. bacterial spoilage), physiological metabolites (e.g. high energy phosphagens, lactate), degradation by-products (e.g. rancidity products, cadaverine), textural strength, colour and lipid composition, to list a few. Such measures are often used as they present an objective quantification of the variable of interest and are therefore more comparable within and between experiments.

Extending Shelf Life and Quality

Immediately after harvesting, fish begin to change from the 'native' state. This change/degradation can refer to numerous processes, including autolysis (self digestion), protein denaturation, oxidation, and bacterial growth, all which may detract from the eating quality of the flesh. Numerous storage or treatment methods can be employed to reduce the rate of quality deterioration of the tissue, including reduction in temperature, modified atmosphere packing, vacuum packing and smoking, to list a few (Huss, H.H. 1995). This thesis is concerned with temperature and harvest effects upon quality deterioration.

Refrigerated and Frozen Storage

By lowering the temperatures at which the flesh is stored the period before spoilage occurs can be prolonged and the shelf-life of the product extended. Lowering of the temperature of a perishable good reduces the thermal energy of the system. Rates of chemical reactions are lowered due to depressed chemical kinetics and deteriorative processes such as oxidation, enzymatic activity and bacterial growth are retarded.

The freezing of food products occurs when storage temperatures are reduced below their freezing point, minimising the thermal energy of the system while also converting a large proportion of associated water to ice. These processes serve to reduce the volume of the aqueous phase that could support microbiological activity and chemical reactions. The process of freezing involves an initial removal of latent heat from water within the system to a point where the nucleation of ice crystals upon a seed occurs. Following nucleation, ice crystals propagate as heat is released from other water molecules, or as solutes migrate from the region of crystal growth (crystals are only formed from pure water) culminating in a phase transition from liquid to solid. In the frozen state, rates of change are greatly reduced and the storage life of the product is greatly enhanced. However, in the frozen state, physical, chemical and enzymatic changes are still able to occur eventually rendering the tissue to an undesirable state. Oxidation of lipids, denaturation of proteins, enzymatic activity and ice crystal dynamics are all capable of reducing the quality of the frozen stored product (Reid, D.S. 1997).

Quality Benefits of Rested Harvesting

Within the literature, harvesting of fish by rested methods has identified many product quality benefits over fish harvested by conventional means. Rested harvesting results in higher concentrations of ATP, with lower concentrations of its metabolic breakdown products. This phenomenon of an enhanced adenylate energy charge can be interpreted as a quality index known as the K-value of freshness, with low values indicating a 'fresher fish'. In a comparison of low and high stress slaughter methods, stressed fish showed a markedly greater 'K-value', that developed earlier in an ice storage period (Erikson, U. et al. 1997). Additionally, high ATP concentrations and glycolytic stores provide an elevated scope for anaerobic activity post mortem, which serves to extend the period of cellular viability, prolonging the pre-rigor period.

Profiling of post mortem metabolism in newly harvested fish has shown that the pre rigor period is extended when compared to tissue harvested using conventional or high activity harvest procedures (Robb, D. & Warriss, P. 1997; Bosworth, B.G. *et al.* 2007). Associated with the enhanced metabolic state of the tissue, quality benefits including: elevated textural strength; lower rigor tensions; altered colour scores; and reduced

tissue gaping; have all been demonstrated when compared to exercised or conventionally harvested tissue (Jerrett, A.R. et al. 1996; Robb, D. & Warriss, P. 1997; Jerrett, A.R. & Holland, A.J. 1998; Robb, D. 2002; Kiessling, A. et al. 2004; Roth, B. et al. 2006).

The Present Study

The purpose of this study is to investigate frozen storage processes within salmon muscle tissue as affected by two different harvesting methods. The two different harvest methods employed, here termed 'rested' and 'exercised', were selected because of the contrasting levels of activity of the animal prior to, and upon slaughter.

Following euthanisation, the physiological state of the animal was ascertained and tissue samples were excised then entered into sub-zero storage. During this sub-zero storage period the profiling of cellular metabolites including ATP, lactate, glycogen and glucose was undertaken. Profiling of these metabolites served to identify any cellular activity during the storage period. Lipid oxidation products were profiled throughout the storage periods as an indicator of rancidity development within the tissue.

The profiling of cellular metabolites and lipid oxidation products were repeated on tissue stored at various temperatures, which included -19°C, -9°C and -1°C, as well as tissue exposed to a freeze/thaw event followed by refrigerated (+4°C) storage.

Underlying this investigation is the question of whether 'rested' harvesting provides any quality benefits to tissue stored in sub-zero conditions. Potential effects may be associated with the levels of stress and activity that occur in the fish, which will have subsequent effects on the pre-slaughter physiology. Alternatively, associated with the metabolic state of the tissue upon freezing, rested tissue may also prove to be more resilient to deteriorative processes owing to greater viability at the time of freezing. Additionally, exogenous factors may be associated with potential differences between the two treatments, as the active ingredient of the aquatic anaesthetic AQUI-S® is isoeugenol, a potent antioxidant within in vitro lipid systems (Priyadarsini, K.I. 1997).

CHAPTER 2

General Methods and Materials

Experimental Fish

The study species utilised within this project was *Oncorhynchus tshawytscha* commonly referred to as Chinook salmon, or King salmon. Chinook salmon are of commercial significance worldwide and are the only salmonid species cultured as a food commodity in New Zealand. Production figures from 2007 identify national production of 7450t, valued at $37,000,000, derived from sales to local and international markets (SeaFIC 2007).

Experimental fish for this study were collected from Isaac Salmon Farm, Christchurch, New Zealand, a commercial freshwater culture facility with production of around 170t per annum. The fish had been reared in an intensive production facility before collection and transfer to a secondary containment facility setup for experimental harvests. Fish were kept for a period not exceeding two weeks prior to experimental harvest.

Fish and Sample Handling Procedures

Fish Containment Facility

The secondary containment system consisted of two 2000l circular fibreglass tanks. Each tank contained 1700l of flow through water sourced from an artesian bore. Stocking densities did not exceed 25kg/1000l water while water temperature remained at a constant 12-13°C year round. Additional aeration was supplied to each tank by a mains voltage bubbler and air-stones. For addition of anaesthetic (required for the rested harvest technique) each tank was supplied with a dose line and bucket that provided a gravity feed of anaesthetic stock solution into the water inflow. No feed was presented to the fish following transfer to the secondary containment facility.

Harvest Procedures

As experiments within this thesis involved an investigation into the effect of fish harvest procedure on the subsequent storage period, two different harvest methods were utilised. The first was a conventional type harvest, resembling methods currently used in commercial settings. This conventional method will be referred to as an 'exercised harvest'. A second 'best practice' method was then incorporated. This method utilised the aquatic anaesthetic AQUI-S® (AQUI-S NZ Ltd, Lower Hutt, New Zealand). This best practice method will be termed 'rested harvest' throughout.

All fish harvest procedures were conducted with approval of the University of Canterbury Animal Ethics Committee, Approvals 2006/8R and 2006/24R

Exercised Harvest

This method involves collecting the fish from their containment tank using a dip net and transferring them into an ice slurry (0-0.5°C). During this procedure the fish showed typical escape responses and associated high levels of exercise/activity. Once immersed within the ice slurry activity levels of the fish decreased until the fish became handleable. At this point the fish was euthanized using the iki-jime technique.

Rested Harvest

The rested harvest was carried out by gravity feeding a stock solution of AQUI-S® as a 1:40 working solution (anaesthetic: fresh water) into the containment tank, providing an exposure concentration of 20ppm (nominal concentration). Slow addition of the working solution over a period of 10 minutes served to ramp the anaesthetic concentration up over time, preventing any possible adverse reactions from the fish owing to the introduction of the anaesthetic. During this procedure every attempt was made not to disturb the fish. The fish were left within the dosed containment tanks until they had reached stage 3-4 anaesthesia and were unresponsive to handling (a total exposure period of 30-40 minutes). At this point the fish were dip-netted from the tank and immediately euthanized using the iki-jime technique.

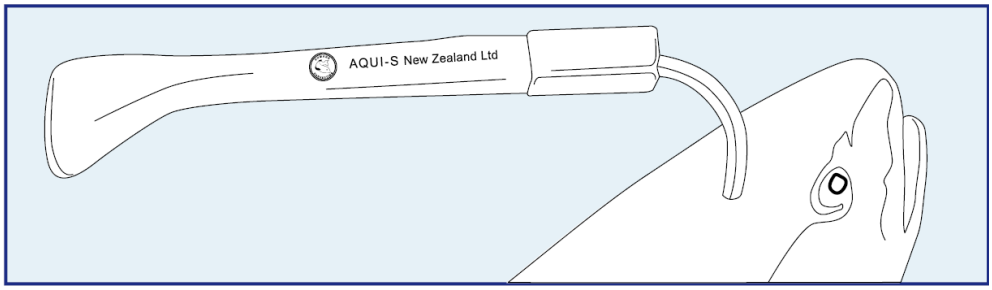

Figure 2- 1: Illustration of the Iki-Jime Brain Ablation Technique (Source: AQUI-S NZ Ltd. www.aqui-s.com)

Blood Sampling

After each fish had been euthanized, 3ml of blood was extracted from the caudal vein (ventral approach) or directly from the heart, using a heparinised 3cc disposable syringe and 18 gauge needle. After extraction the syringe and its contents were immediately placed on ice. Upon removal from ice the blood sample was decanted into centrifuge tubes and spun down to separate the plasma.

Exsanguination

After each fish from both the rested and exercised treatments had been euthanized and a 3cc blood sample drawn from the caudal vein, an exsanguination cut was made across the 'throat' and bulbus arteriosus of the fish. Care was taken not to rupture the transverse septum, so as to prevent bleeding into the visceral cavity. The fish was then placed in a bin of chilled water (at half the acclimated temperature, ≈6°C) and allowed to bleed prior to tissue preparation.

Characterisation of Fish Condition

Morphometric analysis was performed upon each fish harvested to ascertain the condition of the fish. Indicies utilised included condition factor, hepatosomatic index and gonadosomatic index (Love, R.M. 1970). Indicies were calculated as follows CF = weight (g)/fork length (mm^3) x 10,000, HSI = liver weight (g)/fish weight (g) x 100, GSI = gonad weight (g)/fish weight (g) x 100.

Tissue Preparation

The first fillet from each fish harvested within this study was removed, with care taken not to penetrate the gut cavity. A transverse cut along the D-block of muscle tissue (see Figure 2-2) was taken, extending as far back as the anterior origin of the dorsal fin. This cut was then removed from the fillet, the skin layer and any red muscle removed. Successive blocks of WM 3-5mm width (≈2g mass) were then taken, beginning at the anterior origin of the tissue fillet. The excised muscle sample was then freeze-clamped under liquid nitrogen. Following freeze-clamping the tissue samples were either left within liquid nitrogen for time zero analysis, or transferred into the sub-zero storage conditions for profiling over time.

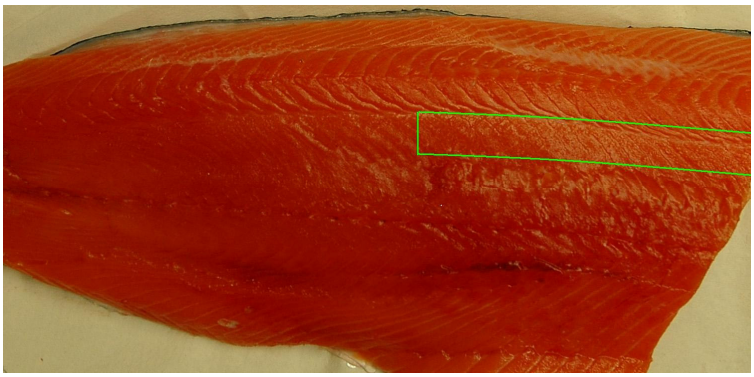

Figure 2- 2: Location of Excised Muscle block (outlined)

Freeze-clamp Method

Freeze-clamping was performed by placing individual tissue slices (2-3g) between two aluminium plates (dimensions: 62mm diameter, 12mm thickness) while immersed in a pool of liquid nitrogen. The aluminium plates used for the clamp had previously been immersed in liquid nitrogen. Following freeze-clamping the tissue block was wrapped in labelled aluminium foil (including a secondary numbered waterproof label) and re-immersed in liquid nitrogen.

The freeze-clamp procedure offers a rapid freezing of the muscle tissue in its entirety. Pre-cooling of the clamps combined with performing the clamp while immersed in liquid nitrogen (b.p. -196°C) provides a frozen surface that facilitates the transfer of thermal heat from the tissue while also preventing formation of an insulating, gaseous layer of nitrogen, providing a rapid freezing of the tissue.

A rapid freezing of muscle tissue, such as occurs in the method described, results in the formation of numerous small ice crystals (Reid, D.S. 1997), with subsequent benefits associated with reduced levels of mechanical damage to cellular structures, and minimised osmotic disturbances to the cellular environment associated with water crystallisation and solute migration during freezing (Santos-Yap, E.E.M. 1995).

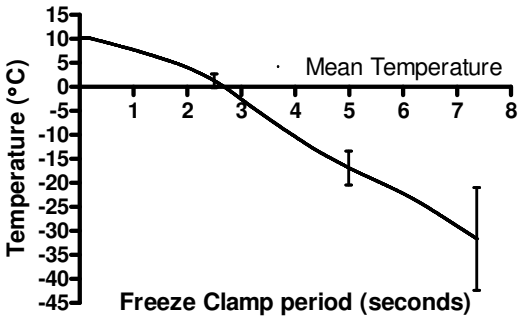

Figure 2- 3: Temperature Change Over Time (mean±sd) of WM Tissue Cuts Utilising the Freeze-clamp Protocol

(n=3, weight: 2.99±0.16g (mean±sd))

The freeze-clamping method described provides a rapid freezing of the fish muscle. Thus, it represents the ideal/preferred method by which to freeze tissue structures (Reid, D.S. 1997). This is both desirable, and employed (to varying extents), within the commercial sector. However, freezing protocols utilised within the commercial sector do not provide freezing rates as rapid as those employed within this research project.

Tissue Sampling

At set time periods during the storage period, tissue samples were randomly selected from the storage unit, removed and immediately placed into N_2(l). The tissue sample was then unwrapped and approximately 0.5–1g of tissue was taken from the square shaped sample. The tissue sub sample was transferred to a pre-cooled mortar and immersed in liquid nitrogen before being ground to a fine powder using a pre-cooled pestle. Liquid nitrogen was repeatedly added to prevent warming of the muscle tissue. A predetermined weight (according to the analysis to take place) was then transferred

to a Nunc Cryotube (Cat # 377267, Nunc Cryotubes®, NY, USA) using a pre-cooled spatula. Further sample preparation procedures can be seen in the sections below.

Metabolite Analysis

Plasma Preparation

Whole blood collected from the fish was decanted into a 1.7ml microtube (Cat # MCT 175-C, Axygen Scientific, CA, USA) following withdrawal from the fish. The plasma component was separated by centrifugation at 10,000rpm for a five minute period. The plasma layer was then removed, transferred to a 1.8ml Nunc tube and then stored at -80°C for up to one week before analysis. Before analysis, frozen plasma samples were thawed under running tap water.

Tissue Preparation

Metabolites (glycogen, lactate, glucose, ATP/IMP ratio and ATP) were extracted from muscle tissue using a perchloric acid tissue extraction. 100mg of muscle tissue was ground and transferred to a Nunc cryo-tube as described previously. Subsequently, 500µl of 6% perchloric acid (PCA) containing 30% methanol was added before further homogenisation (Heidolph Diax 900, Schwabach, Germany, with a type 6G homogeniser fitting) at 26,000rpm for 30 seconds. The sample was then stored at -80°C for no longer than two weeks before analysis. Upon removal from frozen storage the tissue homogenate was thawed under running cold tap water. A 100µl aliquot of tissue suspension was withdrawn via pipette for glycogen analysis. The remaining suspension was centrifuged (10,000rpm, 5min), supernatant withdrawn and then decanted into a fresh microtube. Next, the solution was neutralised with 2M KOH and further diluted with 600µl dH_20. Finally, the sample was vortexed for 30-60 seconds in preparation for lactate, ATP, ATP/IMP and glucose analysis.

For isoeugenol analysis approximately 100mg of WM, ground within the mortar and pestle and under $N_2(l)$, was transferred to a Nunc tube before addition of 900µl reagent grade ethanol. The suspension was then homogenised with the Diax unit and type 6G homogeniser fitting. Following homogenisation the suspension was

centrifuged at 10,000rpm for 10 minutes, the supernatant retained and placed onto ice awaiting analysis.

Glucose Analysis

Glucose was determined within both the plasma component of the blood and within White muscle (WM) d-block slices. Undiluted plasma was used directly for glucose analysis.

Determination of glucose concentration within the plasma and tissue extract was performed using a Roche Gluco-quant Glucose/HK Assay kit (Cat # 11447521 216), Roche, Mannheim, Germany) adapted for use in a bench-top assay. 400µl of Reagent A was mixed with 100µl of sample solution and 500µl of dH_20 before UV Spectrophotometric analysis at 340nm. 50µl of Reagent B was then added for the enzymatic conversion step, followed by incubation at room temperature for 10 minutes, then a final UV spectrophotometric reading at 340nm. Sample readings were zeroed upon a sample blank, while the glucose concentration was calculated by relating sample readings to those determined by analysis of a standard solution (.25g/L).

Principle of Assay:

$$(1) \quad \text{Glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{Glucose-6-phosphate} + \text{ADP}$$

$$(2) \quad \text{Glucose-6-phosphate} + \text{NADP}^+ \xrightarrow{\text{G-6-PDH}} \text{gluconate-6-phosphate} + \text{NADPH} + \text{H}^+$$

HK – Hexokinase
G-6-PDH – Glucose-6-phosphate dehydrogenase

The enzymatic reactions produce stoichiometric concentrations of NADPH. This concentration of NADPH can be measured spectrophotometrically at 340nm.

Lactate

Determination of the plasma lactate concentration required dilution of the plasma in the ratio 1:3 (plasma: distilled water). The diluted sample was then vortexed and analysed immediately. The tissue lactate concentration was determined from the supernatant retrieved from the PCA extraction.

Determination of lactate concentration within the plasma and tissue extract was performed using a commercially available enzymatic assay kit. The Roche L-lactate assay kit (Cat # 10 139 084 035, Roche, Darmstadt, Germany) was initially adopted, but was later replaced (as supplies were discontinued) with Megazyme L-lactic acid assay kit (Cat # KLate, Megazyme, Wicklow, Ireland). Analyses were carried out in 1ml disposable cuvettes as per the instructions of the kit. Sample readings were zeroed upon the sample blank, while the lactate concentration was calculated by relating sample readings to those determined by analysis of a standardised solution (.15g/L).

Principle of Assay:

$$(1) \quad \text{L-lactate} + NAD^+ \xrightarrow{\text{L-LDH}} \text{Pyruvate} + NADH + H^+$$

$$(2) \quad \text{Pyruvate} + \text{L/D-glutamate} \xrightarrow{\text{GTP}} \text{L/D-alanine} + \text{2-oxoglutarate}$$

L-LDH – L-lactate dehydrogenase
GTP – Glutamate-pyruvate transaminase

The first reaction involves the oxidation of L-lactate to pyruvate, reducing NAD^+ to NADH. The second reaction acts to remove the reaction product pyruvate, creating an equilibrium that favours NADH and pyruvate production. The resulting concentration of NADH (stoichiometric to L-lactate) can be measured spectrophotometrically at 340nm.

Glycogen

Muscle glycogen was extracted using the PCA extracted sample outlined previously. Determination of tissue glycogen concentration was performed using a modification of the Keppler and Decker (1974) method. Within a 1.7ml microtube tube, 100µl tissue homogenate (un-neutralised, undiluted) was transferred followed by 50µl of 1M $KHCO_3$. 500µl of acetate buffer containing 2mg/ml amylglucosidase enzyme was then added to the neutralized homogenate before the sample was incubated at 37°C for two and a half hours. The incubation was then halted by placing the samples on ice before centrifugation at 10,000rpm for five minutes. The glycogen concentration of the incubated solution was identified by determining the glucose concentration

liberated during the enzymatic conversion step. These 'glucosyl units' were determined by the commercial glucose assay kits as described previously.

Glycogen content within the muscle was calculated by subtracting the initial glucose concentration of the sample from the glucosyl unit concentration determined following the enzymatic degradation of glycogen. The net glucose concentration identifies the glycogen/glycosyl unit concentration of the tissue.
Principle of Assay:

$$(1) \quad \text{Glycogen} \xrightarrow{\text{Amyloglucosidase}} \text{Glucose}$$

Enzymatic conversion of glycogen to glucose with the enzyme amyloglucosidase allows the quantification of glucose via the Roche Gluco-quant Glucose/HK Assay kit. Converted glycogen is expressed as glucosyl units.

Adenosine 5'triphosphate Determination

Adenosine triphosphate (ATP) concentrations within the WM were determined using the Enliten® (Cat#FF2021, Promega Corporation, WI, USA) assay kit. Assay preparation involved diluting 100µl of the neutralised tissue extract to 1000µl with dH_20. For analysis 90µl of the diluted tissue extract was transferred into a 1ml disposable cuvette containing 430µl of dH_20. 50µl of Luciferase reagent was then added followed by a further 430µl of dH_20 to mix. After mixing, samples were immediately read within a Cary Eclipse Fluorescent Spectrophotometer (Varian Inc, Mulgrave, Australia) using the bioluminescence mode. Bioluminescence settings were: 560nm wavelength; 20nm emission slit width; 150ms reading duration; and 600mV sensitivity. Sample measurements were zeroed on dH_20 and extrapolated on a standard curve determined using standardised solutions (0.5-50µg/ml) analysed according to the procedure described.

Principle of Assay:

$$(1) \quad \text{ATP} + \text{d-luciferin} + O_2 \xrightarrow{\text{Luciferase}} \text{Oxyluciferin} + \text{AMP} + \text{Pyrophosphate} + CO_2 + \text{light (560nm)}$$

In the presence of ATP and luciferase, luciferin is oxidised to oxyluciferin. Light produced during the oxidation of luciferin is proportional to the amount of ATP in solution (when ATP is rate limiting), and can be measured at 560nm.

ATP/IMP Absorbance Ratio

Measurement of the ATP/IMP absorbance ratio was performed as a modification of the method outlined in Korhonen et al. (Korhonen, R.W. et al. 1990). 300µl of the diluted and neutralised tissue perchloric extract was further diluted with dH_20 to give a total volume of 1ml. Spectrophotometric absorbance measures were then taken within quartz cuvettes at 258nm and 250nm. The ATP/IMP ration was then calculated by dividing the absorbance reading at 258nm (representing ATP) with that at 250nm (representing IMP).

Cut Surface pH Determination

Cut Surface pH measurements were performed using a Sensorex Combination pH electrode (Model: 450-C, Sensorex, Garden Grove, CA, USA) connected to a Radiometer pH meter (Model: PHM84, Radiometer, Copenhagen, Denmark). The pH probe was calibrated while set at room temperature against pH 7.0 buffer solution. All cut surface measures were taken on the freshly exposed cut muscle surface of the D-block (see Figure 2-4), with the peak pH value displayed recorded as the final value.

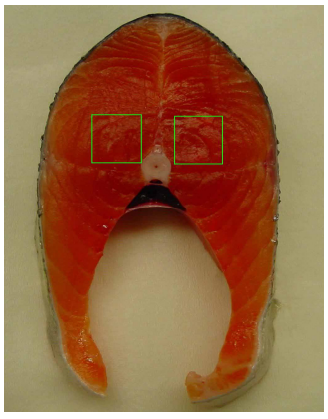

Figure 2- 4 : Location of the D-Muscle Block (outlined) on the Transverse Plane

Lipid Oxidation Product Analysis

Lipid per-oxidation was measured by the TBARS (Thiobarbituric acid reactive substances) assay. The assay is a modification of the Draper et al. method and employs reverse phase HPLC (Draper, H.H. et al. 1993). In this assay aldehydic lipid breakdown products related to MDA (malondialdehyde) react with TBA (2-thiobarbituric acid) to form a TBA-MDA adduct, which can be quantified using fluorometric detection.

Sample preparation involved transferring 200mg of ground WM issue to a 1.8ml Nunc tube. 1.8ml KCl (0.1M) solution containing 10mg/ml EDTA was added to the Nunc tube followed by 40µl of 20mg/ml BHT (butylated hydroxytoluene). The tissue in solution was then homogenised under the Diax 900 homogeniser for 30 seconds before being frozen stored at -80°C, for a period not exceeding two weeks, prior to analysis. On the day of analysis, the frozen sample was removed from storage and thawed under running tap water. 100µl of the tissue suspension was transferred to a 1.7ml microtube, with addition of 10µl BHT and 50µl phosphoric acid (0.15 M). The solution was vortexed before addition of 50µl TBA (42mM) followed by capping, inversion and puncture of the cap. The sample was then placed on a heating block for 30min with very gentle shaking. Upon removal, samples were placed on ice to stop the reaction. Samples were then spun at 10,000rpm for 10min at 4°C. The resulting supernatant was diluted 1:6 in ice cold methanol and centrifuged at 10,000rpm for 10min at 4°C. 100µl of supernatant was then transferred to a sample vial for HPLC analysis.

For the -1 and -8°C storage experiments a modification of the above TBARS sample preparation method was used. The initial sample preparation method utilised incorporated a too high concentration of methanol within the sample upon injection. This high concentration of methanol resulted in precipitation of salt upon injection of the sample solution into the mobile phase, resulting in increase delivery pressures observed at the pump. The modified sample preparation method follows.

After removal of the incubated solution from the heating block 800µl of ice cold methanol was added to the solution and then held on ice for 5 minutes. Next, the

solution was centrifuged at 21,000g for 10min at 4°C. After centrifugation 100µl of the supernatant was added to a fresh microtube and diluted with 100µl 50mM NaH_2PO_4 (pH 6.8). The solution was centrifuged at 10,000g for 5min at 4°C. 100µl of the supernatant was then transferred to a sample vial for HPLC analysis.

Table 2- 1: Details of the Mobile Phase and HPLC Settings for TBARS Analysis

Column	RP-18, 4.6x50 mm C18 Allguard Column	Injection Vol.	10 µl
Mobile phase	45% (v/v) methanol, 55% 50mM NaH_2PO_4 pH 6.8		
Flow rate	1 ml/min	Florescence Detection	Ex: 525nm Em: 550 nm
Column Oven	35°C	Gain	x4
		Sensitivity	Medium

TBARS concentrations in all samples were quantified by comparison to a standard curve constructed using suitable concentrations of 1,1,3,3-tetramethoxypropane (Malondialdehyde or MDA) as the standard. Solutions of MDA, concentration 0, 1 and 3, or 1, 3 and 6 µM were made up, and prepared for analysis as per the methods employed for the sample suspension. A new standard curve was constructed each day of analysis with measurements taken in duplicate or triplicate.

Principle of the Assay:

(1) Thiobarbituric acid + Malondialdehyde $\xrightarrow{\Delta}$ 2:1 TBA-MDA adduct

Reaction of malondialdehyde or other aldehydic oxidation product with thiobarbituric acid forms a fluorescent adduct. This fluorescent adduct can then be measured by detection at 550nm following excitation at 525nm.

Isoeugenol Analysis

The isoeugenol residue within excised WM was determined by fluorescent spectrophotmetry in a method developed developed at Crop and Food Research, Nelson (A.R. Jerrett, S.E. Black and G.J.A. Janssen, unpublished).

Assay preparation involved adding 50µl of the tissue extract to 2950µl with EtOH within a 3ml quartz cuvette. Samples were read within a Cary Eclipse Fluorescent Spectrophotometer (Varian Inc, Mulgrave, Australia) using the fluorometric mode. Fluorescence settings were: 700mV sensitivity; excitation wavelength 266nm; emission wavelength 340nm; 5nm slit width; and 100ms reading duration. Sample measurements were zeroed on MeOH and extrapolated on a standard curve determined using standardised solutions of AQUI-S™ (0-0.5mg.L^{-1}) analysed according to the procedure described.

Protein Denaturation

Protein denaturation was determined by extracting the salt soluble protein fraction from WM in a modification of the Anderson and Ravesi method (Anderson, M.L. & Ravesi, E.M. 1968). Within a highly concentrated salt solution (0.6M NaCl or KCl) myofibrillar proteins (e.g. α-actin, tropomyosin, and myosin chains (Kjærsgaerd, I.V.H. et al. 2006) are predominantly soluble (Jaczynski, J. et al. 2005). Targeting of the myofibrillar (and not sarcoplasmic) component was desired as the myofibrillar component represents 65-80% of the total protein content of the skeletal muscle (Hall, G.M. & Ahmad, N.H. 1997) and has been implicated in the textural degradation of the fillet (Reddy, G.V.S. et al. 1992; Hui, Y.H. et al. 2006)

200mg of WM tissue was added to a high concentration salt solution (HCSS) containing 5ml 0.6M KCl/ phosphate buffer (0.05M) solution containing 0.5% Triton X-100 (Cat # 11 332 481 001, Roche, Mannheim, Germany). The tissue solution was homogenised (Tissumizer, Tekmar, OH, USA) for 30 seconds at 20,000rpm, then centrifuged at 10,000rpm for 20min at 4°C. 40µl of the resulting supernatant was then diluted with 960µl 0.6M KCl/0.05M phosphate buffer for analysis.

Protein determination of the HCSS was then performed using the BCA™ Protein Assay Kit (Cat # 23225 & 23227, Peirce, IL, USA). Within a 1.7ml microtube, 50µl of diluted extract was added to 1ml BCA working reagent. The solution was then incubated for 30min at 60°C in an agitated heating block. Upon completion the solution was placed on ice to halt the reaction before transfer to a 1ml disposable

cuvette and a UV/VIS spectrophotometric absorbance reading at 562nm. Sample measurements were zeroed on dH_20 and sample concentrations were calculated using standardised solutions of Bovine Serum Albumin (Cat#30060-378, Invitrogen, Auckland, New Zealand) (0-250µg/ml diluted in dH_20) and analysed according to the procedure described. All samples were measured in duplicate.

Principle of Assay:

$$(1) \qquad \text{Protein} + Cu^{2+} \xrightarrow{OH^-} Cu^+$$

$$(2) \qquad Cu^+ + 2\text{BCA} \rightarrow Cu^+/\text{BCA soluble complex}$$

In reaction step 1, peptide bonds in protein reduce Cu^{2+} to Cu^+ ions. The number of Cu^+ ions produced is proportional to the amount of protein in the solution. In reaction step 2 each Cu^+ chelates two bicinchoninic acid (BCA) molecules forming a purple coloured solution. This product can be measured by spectrophotmetric analysis at 562nm.

Temperature Control Units

Frozen storage experiments were performed in a Waeco CoolFreeze Kompressor Fridge/Freezer (Model CF40, Waeco, Varsity Lakes, Australia). Storage at -1°C was conducted within an EvaKool Fibreglass Insulated Box (Model B085, Nexburg Pty Ltd, Caloundra, Australia) cooled by a Tropicool Thermoelectric refrigeration unit (Model XC3000A, Tropicool Ltd, Christchurch New Zealand). Storage at 4°C was performed in a water bath controlled with a Lauda Immersion thermostat (Model: MT/2, LAUDA Wobser GMBH & CO. KG, Lauda, Germany). Within the water bath a temporary rack system was fixed so as to hold Falcon™ tubes securely, just above the water level.

Temperature Profiling

Temperature profiling within the temperature controlled storage units was performed using a HOBO Pro (Cat# H08-030-08, Onset, Ma, USA) temperature data logger.

Logging of the core temperature of WM tissue slices was performed using a t-type thermocouple lead (Cat# 158-907, Radiospares, Auckland, New Zealand) connected to a PowerLab 4/25T (Model ML865, ADI Instruments, Sydney, Australia) data acquisition system via a ML312 T-type pod (Cat# PTT0114, ADI Instruments, Sydney, Australia). Temperature profiling of the refrigerated water bath was performed using a Powerlab based MLT404/A RTD-100R Probe (Cat# TRTD0023, ADI Instruments, Sydney, Australia) connected to the Powerlab 425/T data acquisition unit (detailed above) via a ML302 RTD Pod (Cat# PRT0019, ADI Instruments, Sydney, Australia).

CHAPTER 3

The Effect of Frozen Storage -19°C and -9°C on Chinook Salmon (*Oncorhynchus tshawytscha*) White Muscle.

Abstract

Rested and exercised harvesting protocols produced tissue in significantly different physiological states. Rested tissue maintained high metabolic energy stores of ATP and glycogen within the tissue, with low concentrations of tissue and plasma lactate. Exercised tissue exhibited near depleted concentrations of ATP and glycogen and a marked metabolic acidosis. During frozen storage at -19°C, concentrations of these metabolites were unchanged, over the six month period of profiling, within both rested and exercised tissue. During storage of tissue at -9°C, hydrolysis of ATP and glycogen, with no coincident increase in lactate was observed within rested tissue. No significant changes in metabolite levels were observed within exercised tissue, owing to the lack of metabolic energy stores. During -19°C and -9°C storage, harvest treatment was seen to have no significant effect on lipid oxidation processes. However, harvest treatment did have a significant effect on the rate of protein denaturation over six month's storage at -19°C. Results from this investigation suggest that the utilisation of rested harvest protocols has no effect on lipid oxidation rates, but does have an effect on protein deterioration processes, during frozen storage.

Introduction

As outlined in Chapter One, frozen storage of fish tissue helps extend the shelf life period of the product by reducing (bio-)chemical rates of reaction, and retarding bacterial and oxidative attack. However, deteriorative processes such as the oxidation of lipids and

denaturation of protein can still occur in frozen stored fish, limiting the shelf life of fatty fish to approximately four months when stored at -18°C (Johnston, W.A. et al. 1994).

Freezing and frozen storage act to invoke numerous chemical and physical stresses upon the tissue. Ice crystal formation, changes in solute concentration and dehydration all alter the native structure of the tissue. These stresses result in a deterioration of the protein fraction of the muscle tissue where the tertiary structure of the protein molecules is altered or denatured (Xiong, Y.L. 1997). Oxidative damage to the lipids is another major deteriorative pathway within frozen stored fish tissue. The oxidation of lipids results in decomposition of lipid hydroperoxides to volatile aldehydes and ketones. It is these volatiles that result in rancid off-flavours and aromas within the tissue (Hui, Y.H. *et al.* 2006). Additional to the offensive nature of these volatile aromatics, secondary oxidation products of lipids (including malondialdehyde, propanal and hexanal) have also been shown to interact with and denature various proteins (Santos-Yap, E.E.M. 1995). Oxidation of the protein components of the muscle and the formation of protein carbonyls is also implicated in the deterioration of frozen stored fish muscle (Kjærsgaerd, I.V.H. *et al.* 2006). It has been suggested that protein carbonyls may elicit free radical intermediates that react with protein or lipid molecules and form aggregates, resulting in the further denaturation of proteins (Santos-Yap, E.E.M. 1995).

As identified, numerous processes act to cause quality deterioration within frozen stored fish and salmon, whether acting independently or through interaction. The degradation of the tissue, associated with the oxidative and conformational changes mentioned above are often linked to the loss of the water holding capacity of the tissue, resulting in a leaked exudate within thawed fish tissue, which the consumer finds unattractive (Huss, H.H. 1995). Also associated with these physical and chemical changes outlined, the sensory attributes (including texture, odour and succulence) of previously frozen seafood are deemed inferior to that of fresh product (Li, B. & Sun, D.-W. 2002). Thus, thawed/frozen fish is usually viewed as a less desirable product than fresh fish.

The following experimental protocols were employed to investigate whether the method of harvest has any effect on rates of quality deterioration within frozen stored Chinook salmon WM. Rested and exercised harvest methods were utilised, presenting variables associated with the level of stress and activity experienced by the fish immediately prior to euthanisation. Indicators of quality, including the metabolic state of the animal, were profiled immediately following harvest and throughout the storage period. These results were then coupled to quality indicators associated with lipid oxidation and protein denaturation. Combined, these analyses will help to determine whether the physiological state of the animal at the time of harvest has any effects on select degradation processes within the frozen stored tissue.

Following the harvest procedures, blocks of WM tissue were rapidly frozen before being entered into either -19°C or -9°C storage. As presented in Table 3-1, during the freezing of fish tissue, the volume of water converted to ice is temperature dependent, where at temperatures between 0°C and -5°C there is a rapidly increasing volume of ice crystals (between 10 and 80% ice crystals) as temperatures decrease. As temperatures decrease further, the increase in ice volume is more gradual, until -20°C, where ≈90% of the water is converted to ice.

Temperature (°C)	***% unfrozen water***
-1	92
-2	48
-3	33
-4	27
-5	21
-10	16
-20	11

Table 3- 1: Relationship between Storage Temperature and Fraction of Ice Converted to the Frozen State within Frozen Stored Cod (*Gadus morhua*) (James, S.J. 2000).

-19°C was selected as a storage temperature as this represents a temperature with a near maximal volume of ice. It also represents the temperatures typically maintained in a domestic fridge/freezer unit, and potentially utilised within industry. -9°C was selected as it is an intermediate temperature between that of a typical frozen storage unit, and unfrozen temperatures. At -9°C any fluctuations in temperature will result in a change in

the volume of free water/ice crystals, and thus this represents a sub-optimal storage temperature which may be experienced during inadequate frozen storage.

The liquid nitrogen freeze clamp procedure was used as it provides an instantaneous 'fast' freeze, leading to the formation of numerous small ice crystals, and represents the ideal/preferred method by which to freeze tissue structures (Reid, D.S. 1997). Measures of secondary lipid oxidation were performed as they represent degradation products that are associated with rancidity – a major deterioration pathway within salmonid species. Denaturation of myofibrillar proteins was assessed as this is associated with the degradation of protein functionality and textural strength of the fish tissue (Reddy, G.V.S. *et al.* 1992; Xiong, Y.L. 1997; Hui, Y.H. *et al.* 2006). Analysis of metabolites throughout the storage period was performed to identify whether cellular processes occur within the frozen stored tissue.

Materials and Methods

Experimental Fish

Experimental fish that were harvested for -19°C storage were collected from Isaacs Salmon Farm in late January 2007 – concurrent with the peak harvest season for the facility. Once collected, the fish were transferred to the secondary containment facility set up for experimental harvests, conducted in early February. Fish used in the second experiment, involving the storage of fish at -9°C, were collected from Isaac Fish Farm in October 2007. Fish were transferred from the commercial raceways to the secondary containment facility for harvest seven and nine days post transfer.

Harvest Procedures and Fish Handling Procedures

All fish harvest procedures were conducted with the approval of the University of Canterbury Animal Ethics Committee, Approvals 2006/8R and 2006/24R.

For both the -19° and -9°C storage experiments, fish were harvested using 'rested' and 'exercised' methodologies, performed as described in Chapter Two. In both experiments,

fish were placed in one of two tanks – with each tank specified to either the rested, or exercised, harvest treatments. For fish utilized in the -19°C experiment, two fish from the rested treatment were harvested every three days, allowing remaining fish to recover from previous anaesthetisation. Two fish from the exercised harvest were collected the day following, with repeat exercised harvests following three days after, allowing remaining fish to recover from activity associated with the previous harvest event. Fish continued to be harvested in this manner until a sample size of n=7, per treatment, had been attained.

In October, fish were collected for storage at -9°C. Rested fish were harvested seven days post transfer and exercised fish harvested nine days post transfer. In both cases, seven fish were harvested at once, giving n=7 per treatment.

Fish handling procedures, including euthanisation, exsanguination, transport and tissue preparation, were performed as outlined in Chapter Two.

Tissue Storage

The small 2-3g cuts of tissue from each fish sample were held separately in small plastic bags. Collective fish samples comprising a treatment were held within small one litre high-density polyethylene containers. Tissue samples were frozen stored within the Waeco portable freezer unit, utilised in both the -19°C and -9°C storage experiment. The portable freezer unit was kept in a 15°C temperature controlled room to eliminate any fluctuations in ambient temperature.

Sub-sampling of Tissue

At set sampling time points during the frozen storage period, tissue samples were removed and placed within liquid nitrogen, before preparation and analysis.

Analytical Methods

Analytical methods used within this experimental set can be seen in Chapter 2. The assay kits used to determine metabolite concentrations are listed below.

Glucose Analysis: Gluco-quant Glucose/HK Assay kit (Cat # 11447521 216), Roche, Mannheim, Germany).

Lactate Assay: -19°C Storage - The Roche L-lactate assay kit (Cat # 10 139 084 035, Roche, Darmstadt, Germany). -9°C Storage – Megazyme Lactic Acid Kit (Catalogue number K-Late).

ATP Assay: Enliten® (Cat#FF2021, Promega Corporation, WI, USA).

Protein Determinations: BCA™ Protein Assay Kit (Cat # 23225 & 23227, Peirce, IL, USA).

Temperature Profiling

Temperature profiles within the cold storage units were logged using the HOBOPro internal temperature loggers and placed within the receptacle containing the tissue samples.

Statistical Analysis

Comparison of the post harvest condition of the fish harvested using the two different harvest techniques was performed using a Student's t-test. Analysis of metabolic, oxidative and protein degradation profiles, compared between treatments and analysed over time, were tested using a Two Factor ANOVA (repeated measures) with a Bonferroni Post test enabling comparison between treatments and identification of significant changes in the data over time. Within the rested treatment of the October (-9°C) experiment, missing values from the experiment (as n=6 rather than n=7) were replaced with mean values from each sample period within the treatment, thus allowing comparison using Two Factor ANOVA.

Results

Post Harvest Condition

The condition of post harvest fish utilised in the experimental set presented is outlined below (Table 3-2).

Table 3- 2: Morphological and Physiological State of Experimental Fish Post-harvest

(results expressed as mean ± sd. Significant differences, as compared to the rested treatment from each harvest group, are denoted *p<0.05, **p<0.01, ***p<0.001).

	-19°C Storage		***-9°C Storage***	
	January Harvest		**October Harvest**	
Treatment	Rested	Exercised	Rested	Exercised
Sample Size (N)	7	7	6	7
Cut Surface pH	7.54 ± 0.08	6.54 ± 0.04***	7.58 ± 0.08	6.54 ± 0.04 ***
Plasma Lactate (mM)	3.9 ± 2.4	12.6 ± 3.3***	2.8 ± 0.6	5.3 ± 1.0 ***
Plasma Glucose (mM)	3.8 ± 1.0	7.8 ± 4.4*	2.3 ± 0.6	3.5 ± 0.7 **
Weight (g)	3822.7 ± 534.0	3915 ± 765.9 ^{ns}	2345.7 ± 485.3	2853.2 ± 666.7 ^{ns}
Fork Length (mm)	62.1 ± 2.9	59.4 ± 3.3 ^{ns}	53.9 ± 6.3	54.1 ± 3.2 ^{ns}
Condition Factor	1.60 ± 0.20	1.84 ± 0.08 *	1.51 ± 0.23	1.77 ± 0.15 *
GSI	4.98 ± 1.4	4.52 ± 1.98 ^{ns}	0.70 ± 0.31	0.68 ± 0.08 ^{ns}
HSI	1.00 ± 0.10	1.16 ± 0.25 ^{ns}	0.91 ± 0.12	0.87 ± 0.31 ^{ns}
Rigor State	Pre rigor	Early stages of rigor onset	Pre rigor	Early stages of rigor onset
Sex	XX (7/7)	XX (7/7)	XX (6/6)	XX (7/7)

Comparison of the post-harvest physiology of fish from the two different experiments/harvest protocols identified that fish harvested by rested methods, were indeed, in a more rested state. In both experiments, plasma lactate and glucose were significantly lower than fish from the exercised treatments. Cut surface pH values were also significantly higher in rested fish, with exercised fish demonstrating a marked acidosis. No significant differences were seen in morphological indicies (with the exception of Condition factor) of fish when compared to the comparison harvest treatment within the same experiment. The GSI of fish from the January harvest group were markedly higher than those from the October harvest group. This indicates that fish harvested in January were in a much later stage of maturation.

Within the rested treatment of the October harvest group, one fish was excluded from further analysis. The fish excluded was of poor condition, with a broken spinal column and pathological symptoms such as pale flesh, swim bladder oedema, low liver weight and undeveloped gonads. The sample size of the October harvest, rested treatment group, was therefore reduced to six.

Temperature Profiling of Storage Units during Frozen Storage

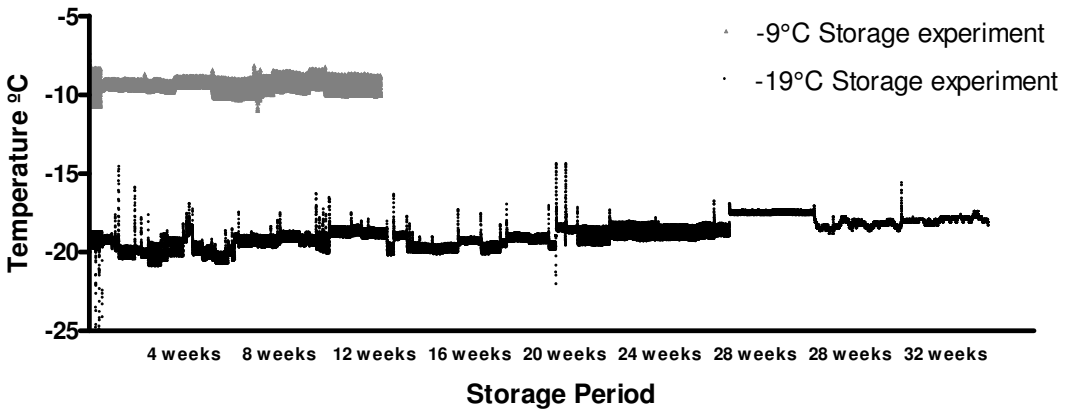

Figure 3- 1: Internal Temperature of Storage Unit during -19°C Frozen Storage.

Internal temperatures of the frozen storage unit are presented in the Figure above. A mean storage temperature of -18.9°C was demonstrated during the -19°C storage experiment, with no major deviations. Small elevations in temperature were observed throughout storage. These related to the opening and closing of the storage unit at set sampling points. A slight upward tracking of temperatures was observed during -19°C storage. This was most likely related to the decreasing mass of tissue in storage (as tissue blocks were removed at each sampling point).

During -9°C storage, a mean temperature of -9.4°C was recorded, with temperatures fluctuating between -8°C and -10°C. Outside of this temperature cycle, no major deviations were identified.

Metabolic Profile

Tissue Adenosine 5'triphosphate

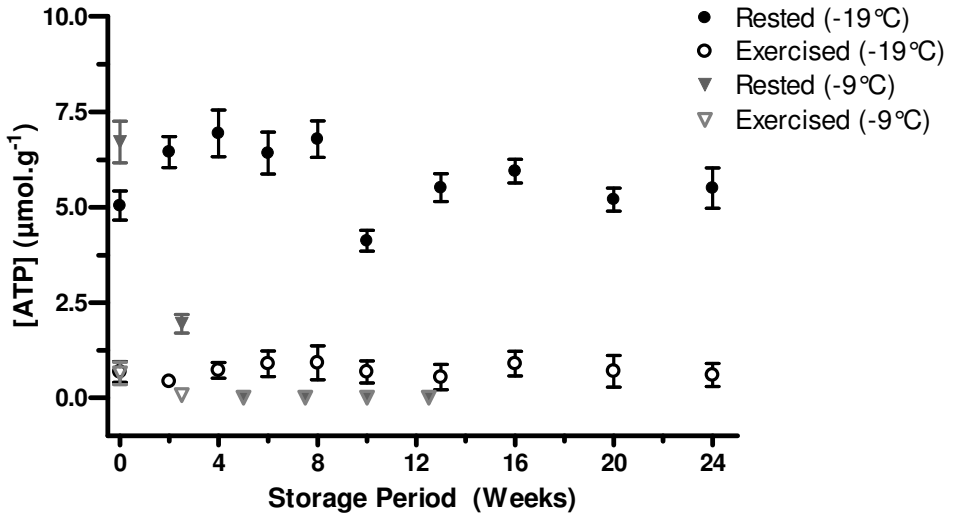

Figure 3- 2: Tissue ATP Concentrations during -19°C and -9°C Frozen Storage (data plotted as mean±s.e.m)

At time zero, ATP stores of rested and exercised tissue, from both -19°C and -9°C, were shown to be highly significantly ($p<0.001$) different. At -19°C, this significant difference was maintained throughout the duration of storage, with no significant deviations from the time zero value identified in either the rested or exercised treatments.

Within -9°C storage, the duration of storage as well as treatment (both $p<0.001$) were seen to have a significant effect on the results. Stores of ATP within the rested tissue were seen to significantly decrease after two and a half weeks storage ($p<0.001$). No significant decreases were seen in ATP stores of exercised tissue, owing to the low starting concentration. After five weeks frozen storage, ATP concentrations in either treatment were below the limit of detection.

Tissue WM Lactate

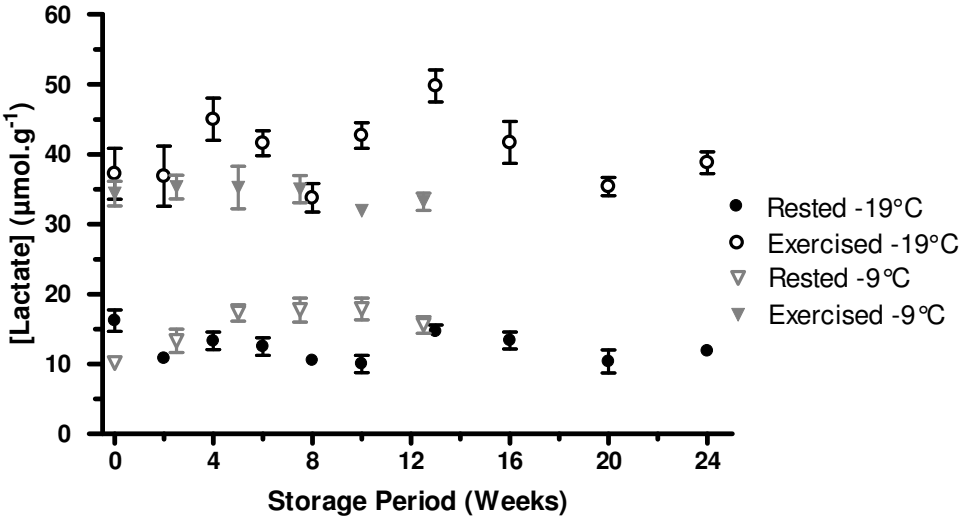

Figure 3- 3: Tissue Lactate Concentrations during -19°C and -9°C Frozen Storage (data plotted as mean±s.e.m)

At time zero, a significant difference between tissue lactate concentrations of rested and exercised tissue was observed within tissue from both the -19°C ($p<0.001$) and -9°C ($p<0.001$) storage. The storage period had no significant effect on the results, at either temperature. This is supported by the Bonferroni post-test that showed no significant deviations from the time zero value throughout storage at both temperatures.

Tissue WM Glycogen

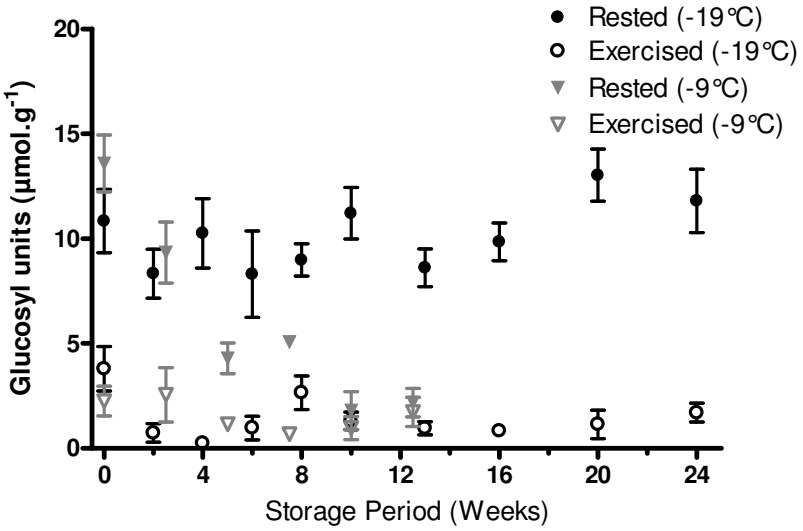

Figure 3- 4: Tissue Glycogen Concentrations during -19°C and -9°C Storage (data plotted as mean±s.e.m)

At time zero, differences in tissue glycogen concentrations were highly significant ($p<0.001$), comparing rested and exercised treatments prior to -19°C and -9°C storage. During -19°C storage, treatment was the only variable that had a significant effect ($p<0.001$) on the results. No significant deviations from the time zero value were seen within either treatment during -19°C storage.

During -9°C storage, both time ($p<0.001$) and treatment ($p<0.001$) had a highly significant effect upon the results. After two and a half weeks storage at -9°C a significant decrease ($p<0.001$) was observed in the tissue glycogen concentration within rested tissue. Tissue glycogen concentrations within rested tissue continued to decrease to low levels during the 12.5 weeks of storage. No significant decreases were observed in the glycogen concentration of exercised tissue, stored at -9°C, owing to low starting concentrations.

Tissue WM Glucose

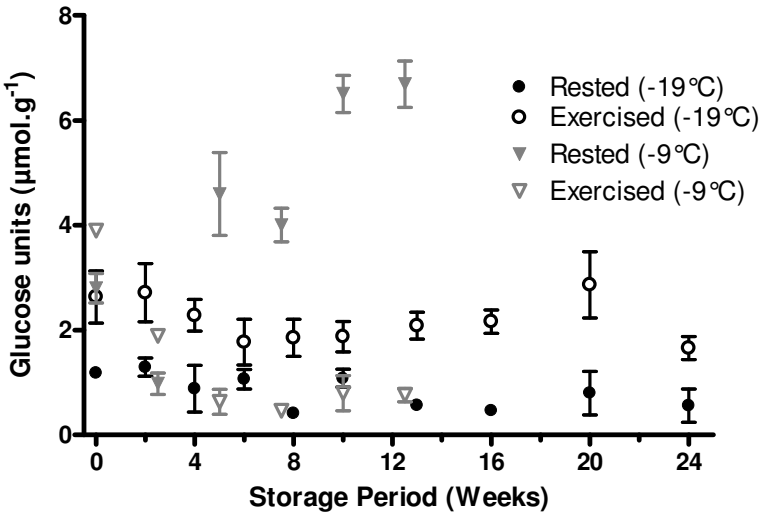

Figure 3- 5: Tissue Glucose Concentrations during -19°C and -9°C Storage (data plotted as mean±s.e.m)

At time zero, concentrations of glucose within the rested and exercised treatments were significantly different in tissue prior to -19°C ($p<0.01$) and -9°C ($p<0.001$) storage.

Within -19°C storage, tissue glycogen stores showed no significant changes throughout the storage period when compared to time zero values. This pattern was observed in both rested and exercised treatments.

At -9°C, duration of storage and treatment were seen to have a significant effect on the results. Within rested tissue a significant decrease ($p<0.001$) in tissue glucose was first observed after two and a half weeks storage. At five and 10 weeks storage, a significant ($p<0.001$) increase in tissue glucose concentrations was observed, when compared to the previous sampling point. These significant increases in tissue glucose coincided with the significant decrease in tissue glycogen stores within rested tissue, observed in Figure 3-4.

Lipid Oxidation

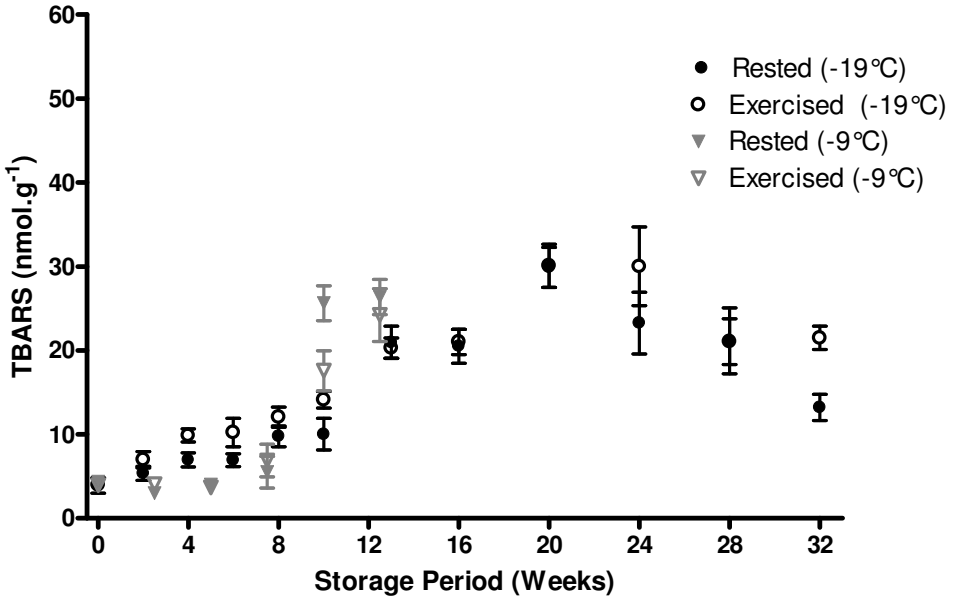

Figure 3- 6: Lipid Oxidation Products in Tissue during -19°C and -9°C Storage (data plotted as mean±s.e.m)

Time zero TBARS values for both harvest treatments within the -19 and -9°C experiments were not significantly different. Within tissue stored at -19°C, only the duration of storage had a significant ($p<0.001$) effect upon the results. Harvest treatment did not have a significant effect on the results ($p=0.074$). However, differences were observed in the time elapsed before a significant increase in the TBARS values (compared with time zero values), after eight weeks storage within tissue from the exercised treatment, and after 10 weeks in the rested treatment. By 13 weeks frozen storage, highly significant ($p<0.001$) increases in lipid oxidation products were observed in tissue from both treatments. TBARS values were seen to peak around 24 weeks frozen storage, and were followed by a significant drop ($p<0.01$) at 32 weeks storage, within both treatments.

During storage at -9°C, the duration of storage was the only variable to have a significant effect upon the results ($p<0.001$). No significant increase in the TBARS values were observed until 10 weeks storage, when compared to time zero values.

Isoeugenol Concentration

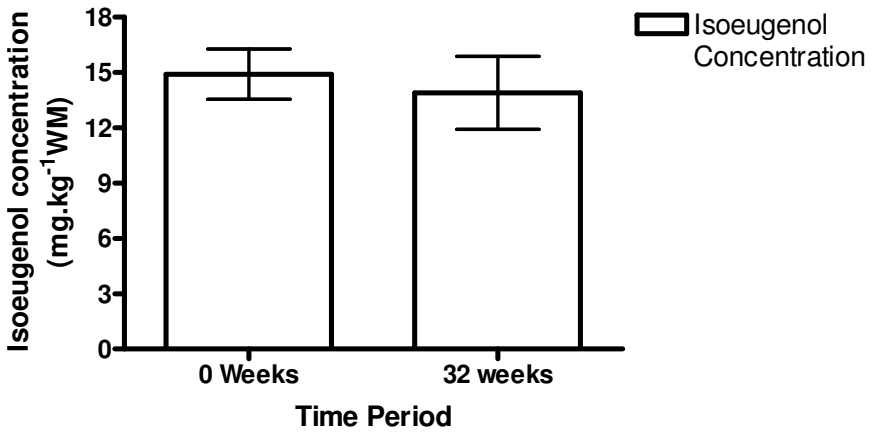

Figure 3- 7: Tissue Isoeugenol Concentrations Before, and Upon Completion of -19°C Storage (data plotted as mean±s.e.m)

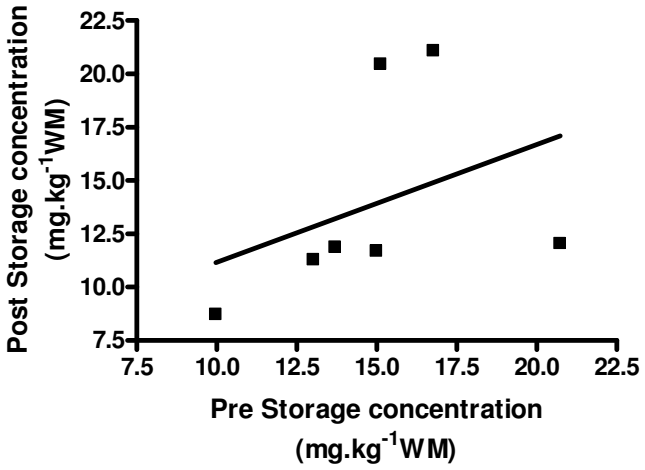

Figure 3- 8: Correlation of Pre and Post Storage Isoeugenol Concentrations within Chinook Salmon WM.

Prior to, and following storage at -19°C, concentrations of isoeugenol within the WM showed no significant differences. The relationship between pre- and post-storage isoeugenol concentrations was investigated with linear regression, identifying a poor, positive correlation (r^2=0.14) between the two sets of values. A strong, negative correlation would provide support for a loss of isoeugenol from WM throughout the storage period.

Protein Denaturation

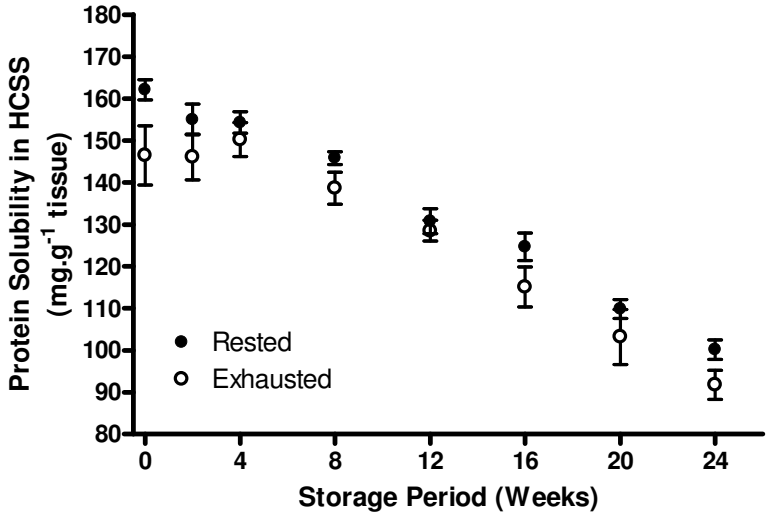

Figure 3- 9: Protein Solubility in a HCSS throughout -19°C Frozen Storage (data plotted as mean±s.e.m)

Two factor ANOVA identified that the duration of storage and harvest treatment has a significant effect ($p<0.001$ and $p<0.01$ respectively) on protein denaturation during -19°C storage. No significant differences between treatments were observed in the time zero protein soluble protein value. Significant decreases from the time zero value were observed after eight weeks in rested tissue and after 12 weeks in exercised tissue.

Rates of protein solubility loss occurring over 24 weeks storage can be expressed as linear regression models. Data points yielded the equations $y=-2.57x+163.1$ (rested tissue) and $y=-2.45x+153.9$ (exercised tissue), with correlation coefficients of $r^2=0.99$ (rested) and $r^2=0.96$ (exercised).

Discussion

Methodology

The intention of the harvest protocol adopted for the -19°C storage experiment performed in January, was to get freshly harvested fish tissue into frozen storage in a minimum of time. However, in doing so the fish had varying levels of prior-exposure to the associated

harvest routine. Rested fish collected had been exposed to between 0-3 anaesthesia and recovery events, while fish from the exercised treatment had also been agitated and exercised between 1-4 times. Such variation in the recent history of the fish had the potential to create wide variation in behavioural responses (i.e. net learning, response to activity outside the tank) of the animal during the harvest and the physiological state of the animal post-harvest. Large standard deviations in plasma lactate and glucose may be explainable by these harvest effects. Following the January harvests, the harvest protocol was amended so all fish within a treatment were harvested at the same time, thus avoiding the potential problems associated with multiple exposure to the harvest protocol.

Metabolites

During storage of WM tissue blocks at -19°C no significant changes in the levels of ATP, glycogen or lactate were observed in either rested, or exercised tissue. This identifies that decomposition of high energy metabolites or cellular activity does not occur during storage at -19°C. Storage of tissue blocks at -9°C identified a decrease of ATP and glycogen stores within the tissue. ATP stores were seen to deplete within five weeks while glycogen decreased to near depleted concentrations after 12.5 weeks. No changes to the concentration of lactate were observed throughout the storage period. These results show that the high energy metabolites ATP and glycogen are unstable during storage and undergo decomposition associated with partial glycolysis.

The decomposition of ATP and glycogen shall be defined ‘partial glycolysis’ as glycogen began depleting as ATP stores fell within the tissue. This suggests both ATP consumption and ATP production were occurring during -9°C storage, although concentrations of ATP were undetectable after five weeks. Tissue glucose concentrations were seen to both increase and decrease as glycogen concentrations were observed to drop. This suggests that the formation of pyruvate was occurring within the tissue. Processes described are termed ‘partial glycolysis’ as lactate accumulation within the tissue was not observed – possibly suggesting enzyme inactivity associated with lactate formation. The basis of this assumption is that the hydrolysis of ATP and glycogen is enzymatic.

The frozen storage of pre-rigor Atlantic cod muscle cuts (≈0.5g), frozen rapidly in an ethanol/dry-ice mix, showed similar stabilities of ATP during storage at -40°C and -80°C over a 12 week storage period (Cappeln, G. et al. 1999). During storage at -20°C, small changes in the ATP concentration were suggested to indicate synthesis and degradation of ATP. No statistical analysis accompanied these results, while a low sample size (n=4) and SD values presented suggest that changes claimed should be treated with caution. For all practical purposes, during storage at temperatures below -20°C, changes in the ATP concentration of pre-rigor frozen stored cod appear to be stable. These results agree with the findings that during -19°C storage of Chinook salmon WM tissue blocks, ATP concentrations remain stable.

In a similar experiment performed on whole, pre-rigor, frozen cod, concentrations of ATP and glycogen were seen to drop, with a coincident increase in lactate within tissue stored at -9°C (Cappeln, G. & Jessen, F. 2001). Tissue stores of ATP and glycogen were seen to deplete after four weeks storage. More gradual changes in ATP, glycogen and lactate were observed during storage at -12°C. The rate of depletion of ATP and glycogen was faster during -9°C storage of Atlantic cod, than was observed during storage of Chinook salmon at the same temperature. The increases in tissue lactate within cod also differed from the stable concentrations observed during storage of Chinook salmon at -9°C.

Muscle tissue derived from other species, including chicken breast muscle and beef neck muscle, frozen pre-rigor, demonstrated low (<0.1) rates of change with respect to ATP and lactate concentrations of the tissue during storage at -10°C (Behnke, J.R. et al. 1973). Metabolic activity (identified by a decrease in ATP and an increase in lactate concentrations) was observed at increasing rates, as storage temperature increased up to -2°C. The results of my study differ from the results of chicken WM or beef neck muscle, possibly owing to differences between enzyme activities in homeotherms and poikilotherms, or structural differences associated with cellular water volumes and functional anatomy of the muscle tissue.

The difference in stabilities of ATP, glycogen and lactate within frozen stored (-19°C and -9°C) Chinook salmon most probably relates to the physical state of the muscle. Fish muscle, including muscle derived from Atlantic cod, tuna and mackerel, have been identified as going through low temperature transitions at temperatures around -11 to -13°C, with additional thermal transitions reported at temperatures ranging from -18°C to -21°C (Jensen, K.N. et al. 2003). It is expected that these temperature transitions indicate the formation of a glassy matrix contained within the muscle proteins (Jensen, K.N. *et al.* 2003). The similarities in glass transition temperatures, independent of the species (and the differences associated with the functional anatomy and composition of the muscle tissue) suggest that similar glass transitions would be observed in the frozen storage of Chinook salmon muscle. The glassy matrix occurs during the propagation and crystalisation of water as storage temperatures decrease. As water in the unfrozen phase becomes more concentrated (with decreasing temperatures) it undergoes a transition to a viscous liquid/amorphous solid, with accompanying low level molecular and kinetic energies (Goff, H.D. 1997). Storage temperatures below the glass transition temperature have been widely suggested to influence food product stability (owing to reduced or inhibited rates of enzymatic reactions and re-crystalisation), although correlations between this state and storage stabilities have not been well defined (Goff, H.D. 1997). Within the data set presented, tissue stored at -19°C showed much greater stability of tissue ATP and glycogen stores than tissue stored at -9°C, which experienced comparatively poor levels of stability. This greater stability of Chinook salmon at storage temperatures below the first (≈12°C) and near the second (-19°C) glass transition zone may well be associated with the physical state of the muscle tissue.

Lipid Oxidation in Rested and Exercised Tissue during Frozen Storage.

No significant differences were observed between harvest treatments during the profiling of lipid oxidation products at either -19°C or -9°C. During storage at -19°C, significant increases from the time zero TBARS value at eight weeks storage for rested tissue and 10 weeks for exercised tissue occurred. During storage at -9°C both treatments observed a significant increase (from time zero values) at 10 weeks.

The significant decrease in TBARS oxidation products after 32 weeks (when compared to 24 weeks) frozen storage is most likely associated with the formation of tertiary lipid oxidation products. Tertiary lipid oxidation products are developed when secondary oxidation products (aldehydes and ketones) react with compounds containing free amino groups (Undeland, I. 2001). Development of these products was not measured, but would explain the decrease in TBARS products. As lipid oxidation processes occur as collective radical and peroxide formation steps, it is expected that further observations of lipid oxidation product development and decline would be identified during prolonged storage. The behaviour (development and decline) of these oxidation products emphasises the need for frequent profiling of oxidation products throughout the duration of storage, thus, enabling a more complete understanding of lipid oxidation processes occurring within the tissue.

Lipid oxidation products were seen to reach higher values in a shorter time frame within tissue stored at -9°C than in tissue stored at -19°C. Similar results have been demonstrated within other salmonid species. Lipid hyper-peroxide concentrations developed faster at -10°C than -20°C, in frozen stored Atlantic salmon (Refsgaard, H.H.F. et al. 1998). Comparisons of my results to those from other species identify that within Atlantic salmon, Coho salmon and pink salmon, rates of lipid oxidation product development, as measured by TBARS, were much lower, particularly when compared to other *Oncorhynchus* species. Pink salmon stored at -20°C for 3 months showed TBARS concentrations of 0.5nmol.g^{-1} MDA (Sathivel, S. 2005) . Coho Salmon stored at -20°C for up to 15 months only showed an increase in TBARS values to 0.08nmol.g^{-1} MDA (Rodriguez, A. et al. 2007). Atlantic salmon stored at -30°C showed a gradual increase in TBARS products peaking at 22nmol.g^{-1} MDA, at ≈35 weeks storage (Hamre, K. et al. 1998). Like my results, the TBARS value of Atlantic salmon stored at -30°C, remained stable then dropped at the next sampling point (≈48 weeks storage). Differences between my results and those from the literature most likely are associated with the form in which the tissue was stored. All results cited involved storage of whole muscle fillets, whereas within my research, profiling was performed on small 2-3g blocks of muscle. Differences associated with the surface area:volume ratio of these different muscle cuts

more than likely had an effect on the observed rates of oxidation, when compared to the other results cited.

In tissue stored at -19°C and -9°C, it took ≈10 weeks before lipid oxidation values had significantly increased from time zero values, despite the differences in storage temperature. It is suspected that variation in the condition of the two harvest (January and October) is the cause for these similar rates of increase. Fish from the January harvest group were at a much later stage of maturation than fish harvested in October, as identified by the comparatively high GSI values. Aging in fish has been found to correlate with elevated levels of oxidative stress, *in vivo*. Within rainbow trout and sea bass muscle tissue of three different age groups, lipid oxidation products (TBARS) were observed to accumulate coincident with declining concentrations of lipophilic antioxidants (including Vitamin E, ubiquinols and ubiquinones) with increasing age (Passi, S. et al. 2004). Similarly in maturing rainbow trout, activities of glutathione peroxidase, catalase and glutathione S-transferase were seen to increase within the muscle tissue, when compared to immature trout (Otto, D.M.E. & Moon, T.W. 1996). These results infer that fish approaching maturation demonstrate elevated levels of oxidative stress, when compared to immature fish. Potentially these changes in the physiological state of the animal with age could have an effect on deteriorative processes in postmortem tissue, resulting in elevated rates of lipid oxidation as was seen during -19°C storage of maturing Chinook salmon.

Throughout the 32 week storage period at -19°C no changes in the concentration of isoeugenol within the tissue were observed. These results, indicate that isoeugenol did not act as a free radical scavenger throughout the storage period.

Protein Denaturation

The solubility of protein within a high concentration salt solution was seen to decrease over the duration of frozen storage at -19°C. The rate of decrease was comparable between treatments, with significant decreases from the time zero value evident after

eight and 12 weeks in rested and exercised tissue respectively. The rate of decrease, in protein solubility, was ≈38% for both the rested and exercised treatments.

Comparable decreases in the salt soluble fraction of fish muscle tissue have been observed in numerous species including hake (*Merluccius merluccius*), lizardfish (*Saurida micropectoralis*), Atlantic mackerel (*Scomber scombrus*) cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*). Of these species, hake fillets and lizardfish fillets showed markedly greater rates of denaturation at -30°C and -20°C (respectively) than those observed in Chinook salmon (as presented) (del Mazo, M.L. et al. 1999).

Storage of Atlantic cod and haddock fillets at -10°C showed a greater rate of solubility decrease than observed above, with extractable protein concentrations dropping to 20% and 33% (respectively) of the original protein concentration (≈120mg.g^{-1}) over 24 weeks storage (Badii, F. & Howell, N.K. 2002). Storage at -30°C resulted in a decrease more comparable to those rates observed within my study, as soluble protein concentrations decreased 30% in cod and 40% in haddock (Badii, F. & Howell, N.K. 2002). The starting protein concentration values of these species were lower than those observed in Chinook salmon, possibly owing to greater delays in tissue processing times or inter-species differences. Frozen storage of mackerel fillets at -20°C showed a slightly faster rate of solubility loss over the first five months of frozen storage, with concentrations of soluble protein decreasing to 50% of their starting value.

Harvest treatment was identified as having a significant effect on protein denaturation. The association between harvest methods and rates of protein solubility loss have not, to my knowledge, been investigated within the literature. However, the effect of harvest treatment on the textural strength of fish tissue following frozen storage has been investigated. Frozen stored (-25°C) Atlantic salmon harvested by either a two step AQUI-S® (rested) protocol or a CO_2 (high activity) protocol, were thawed at +4°C, before texture analysis. Results identified that tissue harvested using the AQUI-S® protocol possessed a significantly firmer texture (as measured by a shear test and blunt compression test), than fillets harvested using the CO_2 protocol (Kiessling, A. *et al.*

2004). As textural strength has been associated with denaturation of myofibrillar proteins (Reddy, G.V.S. & Srikar, L.N. 1991), the results of this study lend support for findings within this investigation.

The formation of low molecular weight ketones and aldehydes, associated with lipid oxidation processes, has been suggested to promote protein denaturation within frozen stored fish muscle (Santos-Yap, E.E.M. 1995; Saeed, S. & Howell, N.K. 2002). Within the results presented, maximum concentrations of TBARS products were observed at 20 and 24 weeks storage. Rates of protein solubility loss through to 24 weeks were constant and able to be expressed as linear equations with very strong correlations associated. Within this investigation, the formation of secondary lipid oxidation products had an indeterminate effect on protein solubility.

Explanations for the loss in protein solubility including aggregate formation and exposure of hydrophobic regions, are all associated with the freezing out of water within the intercellular spaces (Xiong, Y.L. 1997). Possible differences between treatments may relate to the physical properties of pre-rigor proteins within frozen stored tissue, as compared to tissue that has been frozen during, or following rigor. It has been identified that pre-rigor, frozen Atlantic salmon tissue has a tendency for higher breaking strengths than post-rigor frozen tissue, following thawing and over a 10 day cold storage period (Einen, O. et al. 2002). These results suggest that the enhanced textural strength may not be associated with the thaw-rigor phenomenon, which would, it is suggested, only identify elevated levels of textural strength immediately following thawing.

Results from this study suggest that significant differences in protein denaturation within rested tissue are not associated with the lipid oxidation processes within the tissue, owing to the similarity in rates of lipid oxidation between the two different harvest treatments. It is then proposed that pre-rigor muscle tissue is not more resilient (as rates of change were equal between the two treatments) but, rather, possess higher concentrations of soluble protein at the time of freezing. This has been identified within pre-rigor frozen cod, which showed low levels of myofibrillar protein denaturation (as identified by protein solubility scores) when compared to cod frozen in rigor (Love, R.M. 1962). Within my

study, significant differences between the two treatments were not observed at time zero. However, repeated measurement of protein solubility over time identified that a significant effect was present. The low level differences between treatments are probably associated with exercised tissue being in early stages of rigor. Tissue in latter stages of rigor would likely observe greater levels of denaturation (lower soluble protein concentrations). Elevated concentrations of high energy metabolites within the muscle are determinative of the pre-rigor state of the muscle. It appears that this state of rigor, prior to freezing, has an effect on protein denaturation when in the frozen state.

CHAPTER 4

The Effect of Thawing and Subsequent Storage at +4°C, following Freezing, on Previously Frozen Chinook Salmon (*Oncorhynchus tshawytscha*) White Muscle

Abstract

Tissue harvested by rested and exercised protocols provided two separate models with which to investigate metabolic, lipid oxidative and protein denaturation processes during frozen, and subsequent refrigerated storage of Chinook salmon WM.

Upon thawing, frozen stored Chinook salmon underwent metabolic changes that were more dramatic in tissue harvested by rested protocols than tissue harvested by exercised protocols. Abrupt cellular activity identified by the depletion of ATP and glycogen and a gradual increase in lactate was found to occur during thawing at temperatures at, or prior to, -1°C. Upon transfer to refrigerated (+4°C) storage temperatures, lipid oxidation processes were found to proceed at a slower rate in rested tissue compared to exercised tissue. The mechanism of this delayed onset of lipid oxidation within rested tissue is discussed in relation to endogenous and exogenous properties of the muscle tissue. Solubility of proteins in a high concentration salt solution was seen to decrease over time during refrigerated storage. No significant differences were observed between treatments, suggesting equal rates of protein denaturation within rested and exercised tissue during post-frozen, refrigerated storage.

Introduction

As demonstrated in the previous chapter, initial stores of ATP and glycogen are maintained within frozen stored (-19°C) blocks of WM tissue. Associated with these maintained energy stores, low levels of tissue lactate were observed within the tissue.

No significant levels of lipid oxidation and protein denaturation were observed over the first month of frozen storage. As frozen (-19°C) stored rested tissue appears to maintain the energetic stores of fresh, post harvest rested tissue, it is clearly worth investigating the changes that occur within tissue that is removed from the same frozen storage conditions and placed at refrigerated temperatures (+4°C) for further storage. This practice is commonly referred to as freeze chilling (O'Leary, E. et al. 2000). Storage properties of frozen then refrigerated tissue have practical implications and relevance to both the consumer and retailer.

Newly harvested rested tissue maintains a large scope for cellular activity, owing to maintained concentrations of PCr, ATP and glycogen (Black, S.E. *et al.* 2004). Such metabolic processes continue post mortem extending the pre-rigor period beyond 6 hours during iced storage (Azam, K. et al. 1989; Robb, D. 2002). As frozen stored, rested, WM tissue maintains comparable metabolic stores (Chapter 3) to newly harvested rested tissue there is potential scope for metabolic activity upon the thawing of the tissue and its return to the unfrozen state. Such metabolic activity has been demonstrated in thawed, pre-rigor Atlantic cod (Cappeln, G. & Jessen, F. 2001).

Thawing of frozen stored tissue would ideally produce fish tissue in the same state of ‘quality’ that existed prior to the freezing event. However, this is not often the case. During the freezing and storage events, ice crystals form and aggregate. This is caused by both physical and chemical actions within the cellular structure as the osmotic environment of the cell is altered, while mechanical forcing from the crystals themselves can rupture cellular membranes (Reid, D.S. 1997). Such processes act to denature the tertiary structure of the proteins and lyse the cell and its contents altering the microstructure of the tissue from that of its pre-frozen state. Upon thawing, changes in the microstructure of the tissue become evident. Textural degradation and a loss of water holding capacity, caused by the denaturation of proteins and the release of lysosomal enzymes, lessen the quality of the product (Sista, R.V. et al. 1997; Xiong, Y.L. 1997). Changes in odour and flavour can also be evident and are associated with biochemical activity prior to, and during, thawing. Such changes typically become more evident with extended periods of storage (Heen, E. & Karsti, O. 1965).

Following thawing, refrigerated storage provides a low-temperature environment that retards deteriorative processes such as microbial spoilage, lipid oxidation and enzymatic degradation. Storage of the fish tissue at low temperatures will extend the period of quality, when compared to storage at higher temperatures. However, spoilage processes will with time render the fish inedible.

The objective of this chapter is to describe the investigation of changes in frozen tissue stored for a one month period, then transferred to refrigerated (+4°C) storage. Rested tissue, with its maintained metabolic energy stores, is of particular interest owing to the potential for cellular activity upon thaw. A comparison with exercised tissue is performed as it provides a physiological system with near exhausted metabolic energy reserves. Processes of lipid degradation and protein oxidation were tracked to determine whether any differences exist between rates of deterioration in tissue from the two harvest treatments.

Materials and Methods

Experimental Fish

Within this experiment two groups of fish were utilised. Groups of experimental fish for analysis were again collected from Isaac Salmon Farm, Christchurch, New Zealand. For the first experiment, involving tissue exposed to a -19°C storage period followed by refrigerated storage at +4°C, fish were collected during July 2007. Fish were transferred from the commercial raceways to the secondary containment facility (described in Chapter 2) before experimental harvests were performed 7 and 9 days post transfer.

Harvest Procedures and Fish Handling Procedures

All fish harvest procedures were conducted with approval of the University of Canterbury Animal Ethics Committee, Approvals 2006/8R and 2006/24R.

Each experimental group was harvested using ‘rested’ and ‘exercised’ methodologies, performed as described in Chapter 2. Fish handling procedures including euthanisation, exsanguination, transport and tissue preparation were performed as described in Chapter 2.

Tissue Storage

In the first experiment involving frozen then refrigerated storage, small 2-3g blocks of tissue from each fish sample were individually wrapped in aluminium foil and held within capped 100ml polycarbonate Falcon™ tubes. Each tube contained randomly selected tissue samples from each fish within a harvest treatment. Temperature control was provided by the Waeco portable freezer unit set at -18°C and held within a 15°C controlled temperature room. At set sampling time points during the frozen storage period tissue samples were removed and placed within liquid nitrogen before preparation and analysis. After a one month period of frozen storage, the blocks of tissue within remaining capped Falcon tubes were transferred to a water bath held at +4°C where the tissue was allowed to thaw while being held at refrigerated temperatures.

One Falcon tube containing the standard number of tissue slices was set aside for temperature profiling. Prior to the initial freeze clamping of post harvest tissue, a t-type thermocouple was embedded in the core of the tissue and used from temperature profiling of the tissue slices during transfer from frozen to refrigerated storage temperatures.

Sub-sampling of Tissue

At set times during the refrigerated storage period, blocks of tissue from Falcon tubes representing each sampling period were removed. Half of each tissue cut was sectioned and clamped under liquid nitrogen, while the other half was sliced and a cut surface pH measure taken.

Analytical Methods

Analytical methods used within this experimental set can be seen in Chapter 2. The various assay kits used to determine metabolite concentrations are detailed below.

Glucose Analysis: Gluco-quant Glucose/HK Assay kit (Cat # 11447521 216), Roche, Mannheim, Germany).

Lactate Assay: Megazyme Lactic Acid Kit (Catalogue number K-Late).

ATP Assay: Enliten® (Cat#FF2021, Promega Corporation, WI, USA).

BCA™ Protein Assay Kit (Cat # 23225 & 23227, Peirce, IL, USA).

Temperature Profiling

Temperature profiles within the cold storage units were logged using the HOBOPro internal temperature loggers, placed within the receptacle containing the tissue samples. Tissue temperatures were logged using the PowerLab t-type thermocouple setup. The thermocouple lead embedded within a single tissue slice and passing out a predrilled hole within the Falcon tube cap. Storage temperatures within the refrigerated water bath were logged using the PowerLab RTD setup, with the RTD probe immersed within the chilled water. Further details are outlined in Chapter 2.

Statistical Analysis

Comparison of the post harvest condition of the fish harvested using the two different harvest techniques were performed using a Student's t-test. Analysis of metabolic, oxidative and protein degradation profiles were compared between treatments, using a Two Factor ANOVA (repeated measures) test with a Bonferroni Post test enabling identification of significant changes in the data over time.

Results

Post Harvest Condition

The condition of post harvest fish utilised in the experimental set presented is outlined below (Table 4-1).

Table 4- 1: Morphological and Physiological State of Experimental Fish Post-harvest

(results expressed as mean ± sd. Significant differences, as compared to the rested treatment, are denoted *p<0.05, **p<0.01, ***p<0.001).

	Freeze Chill Experiment	
Treatment	Rested	Exercised
Sample Size (N)	6	6
Cut Surface pH	7.55 ± 0.05	6.72 ± 0.02***
Plasma Lactate (mM)	1.4 ± 0.1	7.82 ± 2.7***
Plasma Glucose (mM)	2.6 ± 0.5	7.06 ± 1.1***
Weight (g)	1624.5 ± 377.1	1620.3 ± 433.4
Fork Length (mm)	47.4 ± 3.1	45.8 ± 7.4
Condition Factor	1.50 ± 0.07	1.72 ± 0.41
GSI	0.56 ± 0.12	0.67 ± 0.20
HSI	0.99 ± 0.19	0.86 ± 0.08
Rigor State	Pre-rigor	Early rigor onset
Sex	XX (6/6)	XX (6/6)

Within fish harvested, highly significant differences ($P<0.005$) in the plasma lactate, plasma glucose and cut surface pH values were observed between the rested and exercised treatment groups. Morphological analysis identified that there was no significant difference in the fork length, weight, GSI, HSI or Condition Factor between the two different treatments.

Temperature profiling of storage units and tissue slices during frozen, then refrigerated storage

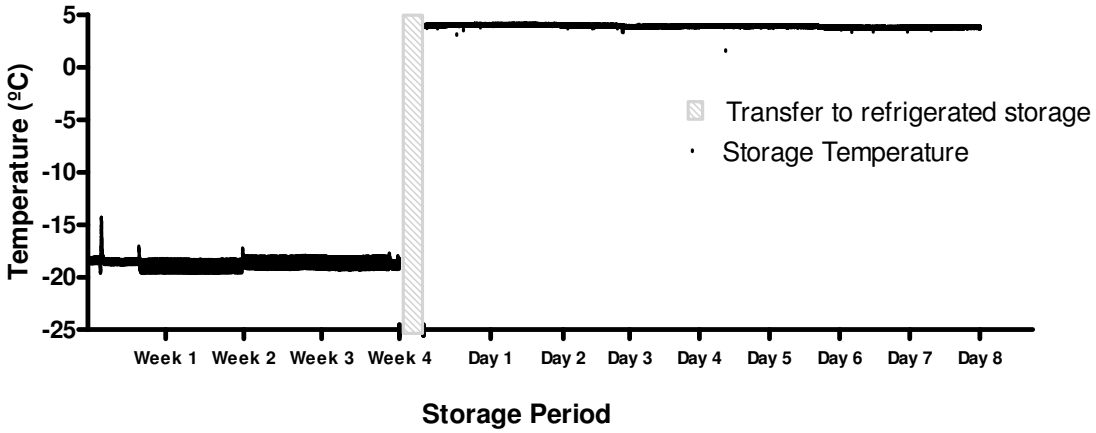

Figure 4- 1: Internal Temperature of Storage Units over Course of Storage Period

Profiling of the temperature controlled storage units identified that during frozen storage an average temperature of 18.8 ± 0.4°C (mean ± sd) was sustained. The temperature of the refrigerated water bath was 3.9 ± 0.1°C (mean ± sd).

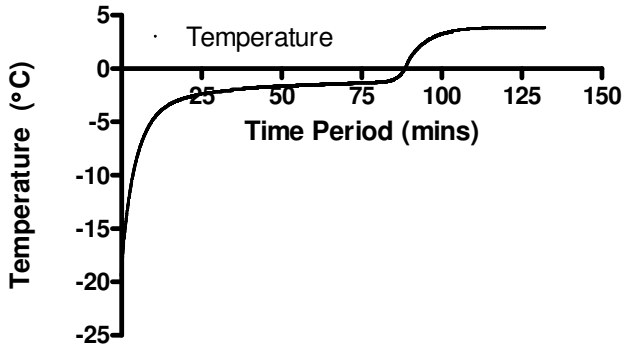

Figure 4- 2: Core Temperature of Tissue slice Following Transfer into Refrigerated Storage.

Upon removal from refrigerated storage, the core temperature of the logged tissue slice shows a rapid (over a period of 10 minutes) warming to temperatures above -5°C. A more gradual warming, extending for a period of 75 minutes, is then observed between -3.5°C and -1.2°C. After reaching -1.2°C, the core temperature of the tissue rapidly rises to +2.7°C and is followed by a more gradual equilibration to the storage temperature (+3.8°C).

Metabolic Profile

Cut Surface pH

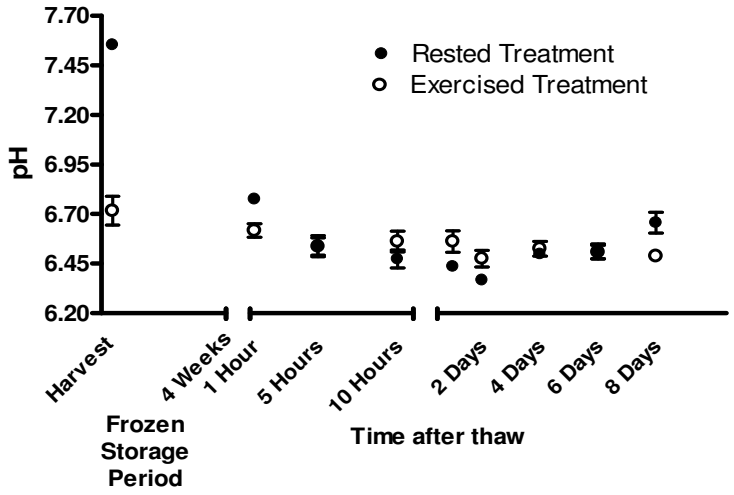

Figure 4- 3: Cut Surface pH Values of Tissue at Time Zero and During Refrigerated Storage.
(Data points are plotted as mean ± s.e.m)

At harvest (time zero) a highly significant difference ($P<0.001$) was observed between tissue harvested by the rested (pH= 7.55±0.05) and exercised treatments (pH= 6.72±0.18). Following removal from frozen storage and placement in refrigerated temperatures, the pH value of rested tissue fell dramatically (an increase in acidity) from that observed at time zero. Comparison by Two way ANOVA of rested harvest values at one hour post transfer (pH= 6.78±0.06) identified a highly significant difference ($P<0.001$) to the time zero value. Following five hours post thaw, there were no significant changes in the pH of rested tissue over eight days. No significant differences in the cut surface pH values of exercised tissue were observed following, and up to eight days after, thawing.

Tissue Adenosine 5'triphosphate

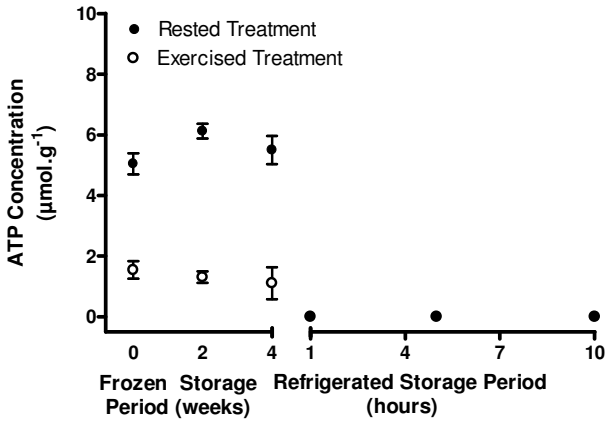

Figure 4- 4: Tissue ATP Stores during Frozen, and Subsequent Refrigerated Storage. (Data points are plotted as mean ± s.e.m)

As previously identified in Chapter 3, tissue ATP concentrations in WM tissue harvested by rested and exercised treatments were significantly different ($p<0.001$) at time zero. Concentrations of ATP in WM tissue from both the rested and exercised harvest treatments remained unchanged (no significant differences) within the four weeks of frozen storage.

Upon thawing, levels of ATP in the muscle rapidly dropped to undetectable levels after one hour at 4°C, where they remained. This represents a highly significant difference ($P<0.001$) to the ATP concentration within rested tissue during frozen storage and a non significant difference with the ATP concentrations within exercised tissue during frozen storage.

Within this data set, concentrations of ATP within the WM tissue were seen to decrease within one hour after transfer. Correlating these findings with data portrayed in Figure 4-2 it can be seen that the loss of ATP occurs at or before -1°C.

ATP/IMP Ratio

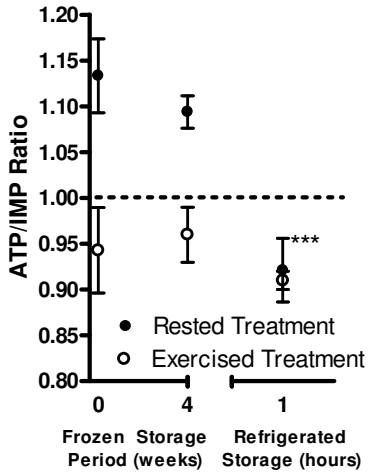

Figure 4- 5: Rigor Index of WM Tissue during the Freeze and Thaw Events. (Data points are plotted as mean ± s.e.m)

The rigor index, expressed as a ratio between ATP and IMP, is a crude indicator of the rigor state of the muscle. Values above one represent muscle in the pre-rigor state, while values below one represent tissue in-, or post-rigor (Korhonen, R.W. *et al.* 1990). Upon thawing the ATP/IMP ratio of rested tissue falls below one. Within the rested tissue, a highly significant difference ($P<0.001$) exists between the four week frozen storage value and the one hour value at refrigerated temperatures. Within exercised tissue, the rigor index of exercised tissue remained below one, with no significant differences between sampling points. Tissue from this experiment could effectively be described as pre-rigor, and in- or post-rigor, muscle for rested and exercised treatments respectively. This finding also correlates with visual observations that muscle from the exercised fish was in early stages of rigor during filleting and tissue preparation steps.

Tissue WM Glycogen and Lactate

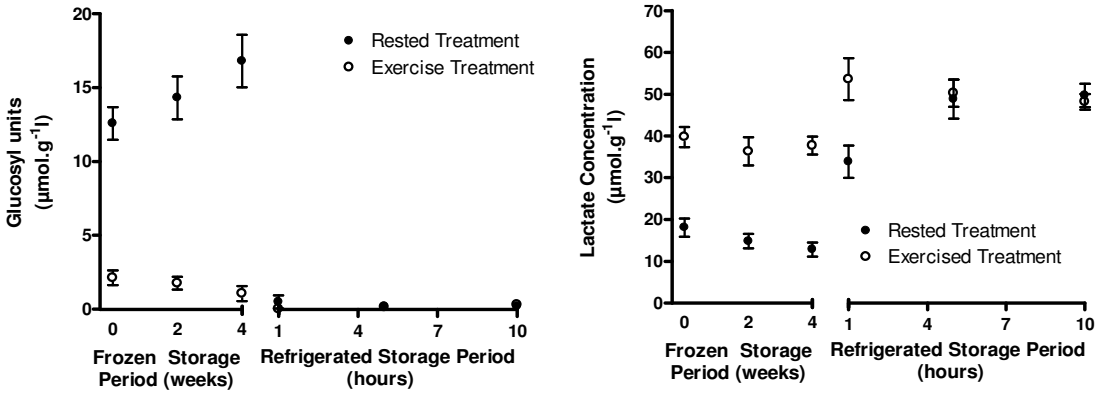

Figure 4- 6: Tissue Glycogen and Lactate Concentrations during Frozen, and Subsequent Refrigerated Storage. (Data points are plotted as mean ± s.e.m)

An observable difference in tissue glycogen and lactate concentrations can be seen between WM from the rested and exercised treatments. Tissue glycogen stores were elevated (12.6±2.7µmol.g^{-1} (mean ± sd)) in rested tissue post harvest (time zero) when compared to the WM from the exercised treatment (2.1±1.2µmol.g^{-1} (mean ± sd)). Tissue lactate concentrations were significantly lower ($p<0.001$) in rested tissue at time zero (18.1 ± 5.4µmol.g^{-1} (mean ± sd)) than exercised tissue (39.8 ± 6.0µmol.g^{-1} (mean ± sd)).

During the frozen storage period, tissue lactate and glycogen remained unchanged (no significant differences) from the time zero value.

Upon transfer to refrigerated temperatures tissue glycogen levels of rested tissue had rapidly depleted by the one hour sampling point, representing a highly significant ($p<0.001$) difference between four week frozen storage values and one hour refrigerated storage values. After one hour at refrigerated temperatures, tissue lactate concentrations increased from those at time zero. After one hour, the tissue lactate concentration of rested tissue had increased to 33.8 ± 9.5µmol.g^{-1} (mean ± sd), representing a highly significant ($p<0.001$) difference to the four month frozen store value. After five hours the lactate concentration value had risen to 48.8 ± 11.8µmol.g^{-1} (mean ± sd), ($p<0.01$) indicating a gradual increase in lactate concentrations over that four hour period.

Within exercised tissue, glycogen stores from both rested and exercised treatments were depleted one hour after transfer (data points overlap in Figure 4-6), representing a significant ($p<0.05$) decrease from the four week frozen storage value. Concurrently, lactate concentrations increased to 53.6±12.3µmol.g^{-1} (mean ± sd) after one hour, representing a significant ($p<0.05$) increase from the four week frozen storage value. No significant changes in lactate concentration were observed in the following sampling points. These results indicate that upon thaw, the tissue is metabolically active, undergoing glycolysis.

Within these experiments tissue glycogen was seen to deplete within one hour after transfer, while tissue lactate was seen to significantly increase one hour following transfer into refrigerated temperatures. Correlating these findings with Figure 4-2 it can be seen that glycolytic activity is evident at temperatures below, or equal to -1°C.

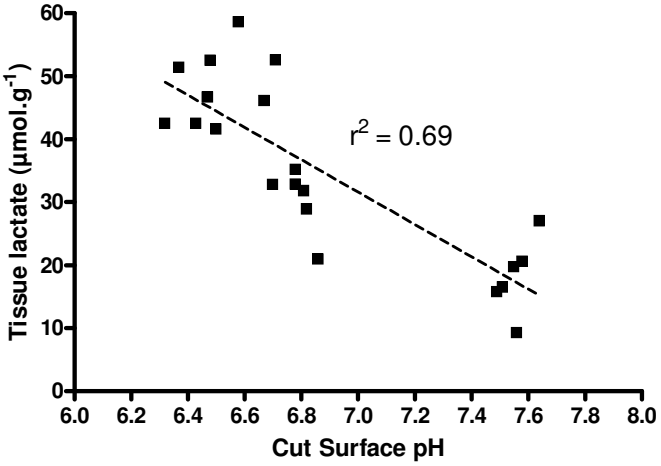

Figure 4- 7: Comparison of Tissue Lactate Concentrations with Flesh pH Values of Rested Tissue Prior to and After the Thaw Event

Comparing tissue lactate and cut surface pH values of rested tissue, before and after thawing, only identified a moderate correlation between the two typically related values. Within the data set, only 69% of the variation observed in tissue lactate was caused by the variation in cut surface pH values.

Tissue WM Glucose

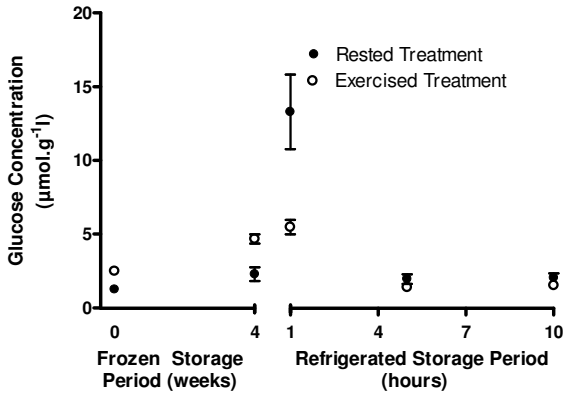

Figure 4- 8: Tissue Glucose Concentrations during Frozen Storage and after Transfer to Refrigerated Storage. (Data points are plotted as mean ± s.e.m)

At time zero, a significant difference ($P<0.001$) existed between glucose levels within rested ($1.2\pm0.1\mu mol.g^{-1}$) and exercised tissue ($2.5\pm0.6\mu mol.g^{-1}$). No significant changes were observed throughout the remainder of frozen storage. After one hour at refrigerated storage temperatures, a spike in the glucose concentration of tissue from the rested ($13.3\mu mol.g^{-1}$) and exercised ($5.5\pm1.2\mu mol.g^{-1}$) treatments was observed. These spikes represent highly significant differences ($p<0.001$) to the respective time zero values for both treatments. After five hours refrigerated storage, tissue glucose concentrations in both treatments dropped to levels $\approx 1\mu mol.g^{-1}$. These results support the evidence of glycolytic activity during the thawing of frozen stored WM.

Within this data set, the spike in glucose concentrations was observed to occur at the same time period as the depletion of glycogen and increase in lactate concentrations. These events all coincided with temperatures of -1°C or less, when correlated with the temperature plot portrayed in Figure 4-2.

Lipid Oxidation Profile

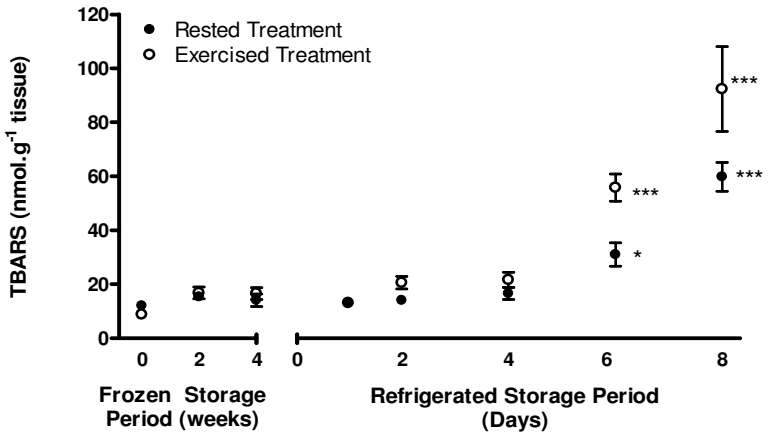

Figure 4- 9: Lipid Oxidation within WM (as measured by TBARS) during Frozen and Subsequent Refrigerated Storage. (Data points are expressed as mean ± s.e.m)

Measurement of lipid oxidation products throughout the profiled storage duration identified that lipid oxidation products increased significantly over time ($p<0.001$). The harvest treatment utilised also had a significant effect ($p<0.01$) upon the results, with exercised tissue showing higher concentrations of lipid oxidation products earlier in time.

At time zero, the concentration of lipid oxidation product was ≈10nmol.g^{-1} with no significant differences between treatments. Following removal from frozen storage, a significant increase in lipid oxidation products was not seen until six days refrigerated storage, with a more gradual increase observed within rested tissue. After six days refrigerated storage, rested tissue contained 31.0±10.6nmol.g^{-1} lipid oxidation products while exercised tissue contained 55.8±12.5nmol.g^{-1} lipid oxidation products. Significance values of the observed increase at day 6 were ($p<0.05$) for rested tissue and ($p<0.001$) for exercised tissue compared with values at time zero.

Protein Denaturation

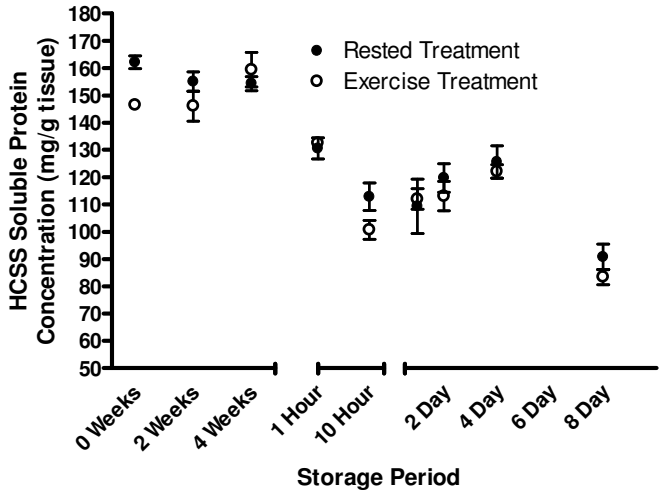

Figure 4- 10: Soluble Protein Concentration (within a HCSS) of WM during Frozen and Subsequent Refrigerated Storage. (Data points are expressed as mean ± s.e.m)

Over the course of profiling, a significant decrease ($p=<0.001$) in protein solubility (and thus, increase in protein denaturation) was observed. The harvest treatment employed was shown to have no significant effect ($p=0.69$) on the results.

At time zero, mean solubility of the WM myofibrillar proteins was 162.10mg/g and 146.49mg/g tissue for rested and exercised tissue respectively. There were no significant differences between these values. For rested tissue a significant decrease in protein solubility was observed after one hour in refrigerated storage temperatures ($p<0.001$), whereas a significant decrease was not observed until 10 hours refrigerated storage within exercised tissue. Solubility values showed great variation, reflected in the large error bars in Figure 4-10.

Discussion

Methodology

As the time periods for sampling within the first freeze/thaw experiment detailed within this chapter were not sufficiently close in time. Thus, the temperatures at which metabolic activity occurred could not be determined accurately. Hence, the experiment was later repeated (refer Chapter 5), with a separate group of fish, in order to more accurately identify the temperatures at which metabolic activity took place.

Utilisation of only one thermocouple (for each thaw experiment) to model the core temperature of tissue slices during warming was probably insufficient to characterise the rate of warming for the population of tissue samples. With hindsight (or more thorough fore-thought) three or more thermocouple embedded tissue slices should have been used to model the warming process.

The denaturation of proteins (which can be measured as a decrease in protein solubility) has widely been cited as the cause for textural degradation in fish products (Santos-Yap, E.E.M. 1995). The protein solubility assay employed within this and the previous chapter does not appear to provide a suitable determinant of protein functionality within the tissue. Large deviations associated with each measurement in time meant that it was difficult to identify significant changes within the tissue. Ultimately, the use of the high-salt protein solubility assay employed only provided a crude profile of protein denaturation, which identified a decrease in protein solubility over time. However this did not identify any potential differences between treatments. Whereas personal observations suggest differences did exist; these are discussed later within this section. Numerous authors have cited the difficulty in objectively measuring, and obtaining significant correlations of, textural changes in fish tissue, owing to variation within the tissue (Santos-Yap, E.E.M. 1995). Within this investigation, it is assumed that the assay utilised also failed to provide a definitive evaluation of protein and textural changes in Chinook salmon during frozen, then refrigerated storage.

Fish condition and physiological state

When comparing fish within experimental designs, it is preferable to compare fish of the same size and condition. Fish from these two harvest groups were of good, comparable condition (no significant differences in CF values). HSI values were moderate-low (≈0.9), but comparable between groups.

Flesh pH values of the rested fish were slightly higher than previously published values of Chinook salmon (Jerrett, A.R. *et al.* 1996; Jerrett, A.R. et al. 2000; Black, S.E. 2002), indicating the rested harvest was well performed, with minimal levels of activity occurring prior to, and during, the harvest event. Plasma levels of lactate and

glucose were low in rested treatments from the two harvest groups, and in agreement with other published results on salmonids (Milligan, C.L. & McDonald, D.G. 1988; Milligan, C.L. & Girard, S.S. 1993; Black, S.E. 2002).

Cut surface pH values of fish from the exercised treatment are comparable to those of exercised/exhausted Chinook salmon within the literature (Jerrett, A.R. *et al.* 1996). Blood lactate values are lower than values cited for rainbow trout and Coho salmon (Milligan, C.L. & McDonald, D.G. 1988; Milligan, C.L. & Girard, S.S. 1993).

Tissue Core Temperature during Thawing

The slow warming of tissue up to -1.2 identifies the zone where the tissue gains latent heat of fusion occurs within the cellular structure. Following the zone demonstrating the latent heat of fusion, tissue is in the unfrozen/thawed state. Hence, the melting point of Chinook salmon WM is equal to -1.2°C. Other research on Chinook salmon identified a freezing point of -1.2°C in WM (Jerrett, A.R. *et al.* 2000), confirming the findings.

Post Thaw Metabolism

During the frozen storage period tissue stores of ATP, glycogen and lactate were comparable to those detailed in Chapter 3. Upon removal from frozen storage, and placement in refrigerated temperatures, the tissue underwent metabolic activity. This cellular activity was evidenced by significant decreases in ATP and glycogen stores and increased lactate concentrations within rested tissue. Significant decreases in tissue glycogen and an increase in tissue lactate was demonstrated within tissue from the exercised treatment.

As shown in Figure 4-6, depletion of glycogen stores result in an increase in lactate concentrations within the tissue. In controlled glycolytic, anaerobic metabolism, each molecular unit of glucose is broken down into two molecular units of pyruavte, with each pyruvate been transformed to a lactate molecule. Within rested tissue, time zero glycogen and glucose stores averaged 14µmol.g^{-1} depleting to zero 10 hours following transfer to +4°C. Tissue lactate concentrations were ≈18µmol.g^{-1}, increasing to 46.6µmol.g^{-1} 10 hours post transfer. Applying the ratio presented to the observed results 14 molar units of glucose (glycogen + glucose) produce 28µmol.g^{-1} of lactate,

which accounts for the ≈28µmol.g^{-1} increase in tissue lactate observed. These calculations identify anaerobic glycolysis was able to occur in typical fashion with rested tissue, and enzymatic processes were unaffected by the frozen storage period.

Pre-rigor, frozen stored (-12°C) Atlantic cod (*Gadus morhua*), has demonstrated a depletion of ATP and glycogen during the thawing in air at 0°C (Cappeln, G. & Jessen, F. 2001). Within their experiment the WM concentration of ATP was seen to significantly increase, with a concurrent significant decrease in glycogen stores prior to concentrations of both falling to undetectable levels. This indicated that during the thaw, ATP is generated from glycolytic activity. No profiling of tissue temperature was performed during the thaw event, thus it is difficult to say at what temperature the tissue underwent this metabolic activity.

Comparing the thaw process of freeze tolerant wood frogs (*Rana sylvatica*) provides suggestions of the metabolic processes that occur during the thawing of pre-rigor frozen stored Chinook salmon tissue. Freeze tolerant wood frogs, through physiological and behavioural adaptations, are naturally able to tolerate freezing. Freezing of their body is aided by the increased concentrations of glucose within circulating plasma, providing controlled nucleation of ice crystals within the extracellular spaces (Storey, K.B. 1997; Storey, K.B. & Storey, J.M. 2004). Upon thawing, numerous enzymes associated with glycolysis, the TCA cycle and adenylate metabolism increase their levels of activity. Within skeletal muscle, the enzymes LDH and CK increased in activity 16% and 72% respectively, while the activity of HK decreased 71% (Cowan, K.J. & Storey, K.B. 2001). These increases in enzymatic activity may support the finding of rapid metabolic activity in thawing Chinook salmon WM, seen within the above experiments. However, elevated cellular activity in the thawing wood frog may be a function of the increased physiological concentration of glucose, which may be toxic to the animal post thaw (Cowan, K.J. & Storey, K.B. 2001), rather than a temperature related increase in activity.

Rested tissue cut surface pH values, prior to, and following frozen storage (Figure 4-3), crudely support the data from tissue metabolites, as a decrease in pH coincides with an increase in lactate and a decrease in glycogen stores. However, upon closer examination (see Figure 4-7) it can be seen that lactate production does not occur in a

strongly linear fashion with the decrease in pH values, as has been demonstrated in elsewhere (Jerrett, A.R. *et al.* 2000; Black, S.E. 2002). This suggests a degree of metabolic uncoupling may occur in reference to the production of H^+ and lactate. Generation of H^+ is known to occur during the hydrolysis of ATP, and thus is an independent reaction from the production of lactate (Hochachka, P.W. 1985). The modest correlation identified in Figure 4-7 may suggest that, during the thaw, these two processes are uncoupled, and occur independently in time. Results from my study are limited and fail to lend strong support to either argument. Therefore, it is recommended that further investigation of this phenomenon occurs, before any stronger conclusions are drawn.

Upon transfer to +4°C the metabolic activity identified occurred in an abrupt manner (within 60 minutes). This metabolic activity occurred much more rapidly than typical post mortem metabolism within newly harvested, rested, teleost WM (Black, S.E. 2002; Bosworth, B.G. *et al.* 2007). In the study conducted by Jerrett (2000), an abrupt increase in metabolic rate was observed during storage of Chinook salmon WM during storage at -1.2°C, a temperature near that at which we observed rapid cellular activity within our thawing tissue. Similarly, the study of Cappeln (2000) showed a rapid glycolytic event in Atlantic cod. The abrupt nature of metabolic depletion at temperatures at or near the freezing point of WM may well be associated with the state of cellular injury within the tissue. During the thawing of frozen stored (-18°C) rainbow trout (*Oncorhynchus mykiss*) a significant leakage of the lysosomal enzymes β-N-acetylglucosaminidase (NAG) was evident, identifying cellular membrane disruption (Nilsson, K. & Ekstrand, B.O. 1995). The rupture and release of cytosolic (and lysosmal) enzymes, associated with the formation and melting of ice crystals during thawing, would create an aqueous environment with high concentrations of metabolic enzymes and their substrates, as well as enhanced concentrations of enzymatic potentiators (such as Ca^{2+}). Such a system would be analogous to the freezing of cellular fluids, where, at and below the freezing temperature of tissue systems, solute concentrations within the unfrozen phase increase (Reid, D.S. 1997). Such a system, if present in practice, could well show high levels of enzymatic activity, resulting in abrupt metabolic activity, in direct contrast to the expected slow increase in enzymatic activity associated with low temperatures and the Q_{10} effect.

Judging from the ATP/IMP and ATP concentration profiles obtained, upon thawing tissue enters a state of rigor, often termed thaw rigor (Dransfield, E. 1996; Cappeln, G. & Jessen, F. 2001; Einen, O. *et al.* 2002). From observation of the post thaw, tissue from the rested treatment was texturally stronger while being more resilient to handling. The tissue did not pull apart with ease, as opposed to tissue from the exercised treatment which was softer, and more friable. Further differences were difficult to identify, owing to the small size of the tissue slices handled.

Lipid Oxidation during Frozen, then Refrigerated Storage.

During the one month frozen storage period, no significant levels of lipid oxidation were identified in tissue samples from either the rested or exercised treatments. However, upon removal from frozen storage a significant difference in the rates of lipid oxidation was observed, with rested tissue showing a slower increase in lipid oxidation products over the eight day period of refrigerated storage.

The effect of freeze chilling on rancidity development is not well discussed within the literature. Freeze chilling of farmed Atlantic salmon (*Salmo salar*), stored at -30°C for three days followed by storage at 2-4°C for five days demonstrated an increase in primary lipid oxidation products to 4.40 meq.peroxide.kg^{-1} (Fagan, J.D. et al. 2003). Lipid hydro-peroxides are a primary lipid oxidation product while TBARS are a secondary oxidation product and so are not directly comparable.

Chilled storage of fresh Coho salmon, at temperatures of 0-2°C, showed lower initial concentrations of TBARS products (≈0.12nmol.g^{-1} MDA) when compared to starting values within my experiment (≈10nmol.g^{-1} MDA), that did not significantly increase until 19 days of storage (at a value of 0.5nmol.g^{-1} MDA) (Aubourg, S.P. et al. 2005). The site, and type of muscle tissue analysed within the mentioned experiment were not detailed. However, the values given are markedly lower than values identified within this study, possibly suggesting differences in the oxidation potential of the tissue and differences associated with the storage regimes.

Thawing of tissue results in cellular decompartmentalisation and leaching of free metal ions (Benjakul, S. & Bauer, F. 2001; Hui, Y.H. *et al.* 2006). Additionally, FFA generation throughout frozen storage occurs as a result of lipase and phospholipase

activity (Reddy, G.V.S. *et al.* 1992; Hui, Y.H. *et al.* 2006), providing a substrate that could potentially be oxidised. Hence, the higher rates of degradation within salmon muscle exposed to a previous duration of frozen storage, as opposed to fresh-chilled salmon muscle are likely explained by the changes that occur in the tissue during frozen storage.

Within minced Atlantic mackerel (*Scomber scombrus*) muscle stored at +4°C, starting TBARS values (≈1nmol.g^{-1} MDA) were again much lower than the starting values within our experiment. However, lipid oxidation products were observed to increase significantly after three days' storage, climbing to values of 38nmol.g^{-1} MDA after seven days. Hence, rates of oxidation appear to be faster in minced mackerel, when compared to small slices of Chinook salmon. Whether this is a surface area, or species related phenomenon, remains undetermined owing to the differences in experimental design.

Retarded oxidation processes within rested tissue have been identified in rested Chinook salmon when stored at +15°C (Tuckey, N.P.L. 2008). However, the ability of rested Chinook salmon, stored at +15°C, to retard lipid oxidation (when compared to a control) was greater than is reported during the frozen, then refrigerated storage experiment presented. This difference in lipid oxidation, suggests that frozen storage may have a detrimental effect on the retardant property that the rested tissue possesses.

The antioxidant properties of fish harvested by rested methods may stem from either an endogenous, or exogenous property of the tissue. The active ingredient of AQUI-S®, the food-grade anaesthetic used within this thesis, is isoeugenol (2-methoxy-4-[(1*E*)-prop-1-en-1-yl]phenol) a potent antioxidant within in-vitro biochemical models (Rajakumar, D.V. & Rao, M.N.A. 1993; Priyadarsini, K.I. 1997; Ito, M. et al. 2005). Isoeugenol has been shown to markedly inhibit iron mediated lipid oxidation, as well as copper mediated low density lipoprotein oxidation LDL. Possible mechanisms of this antioxidant protection relate to the ability of isoeugenol to form complexes with free metal ions, thus maintaining iron and copper ions in a reduced state (Ito, M. *et al.* 2005). Isoeugenol is a highly lipophillic molecule, also providing support for its ability to retard lipid oxidation (Ito, M. *et al.* 2005). Thus, isoeugenol might be an

antioxidant in frozen, then refrigerated Chinook salmon WM tissue, delaying lipid oxidation processes. Endogenous aspects of the fish WM, as affected by the harvest procedure may also have an effect on lipid oxidation during storage.

Electron transport chains within mitochondria are recognized for their ability to produce oxygen radicals within both mammals and teleosts (Wilhelm, D. 2007). Strenuous long and short duration exercise has been shown, in mammalians, to overwhelm ROS detoxification mechanisms, resulting in a decrease in plasma antioxidants with a concurrent increase in blood lipid peroxides (Marzatico, F. et al. 1997). No such experimental evidence is available for exercising teleosts. However, cultured sea bass (*Dicentrarchus labrax*), exposed to a hypoxia-recovery event showed decreased plasma levels of α-tocopherol and ascorbate, with no change in glutathione (GSH). Chub (*Leuciscus cephalus*), during repeat exhaustive swimming events, showed no significant changes in tissue levels of GSH and superoxide dismutase. Fish harvested by the exercised methodology evidence/show high levels of escape behaviour coinciding with a loss of metabolic energy stores that may be associated with a functional hypoxia. Depletion of antioxidant levels such as α-tocopherol and ascorbate or the generation of free radicals within cellular compartments may therefore have a negative effect on storage quality of tissue. Such processes, to my knowledge, have not been described within the literature.

Alternative metabolic processes associated with exercised harvest protocols include the lipolysis of free fatty acids (FFA). The role of triacylglycerol derived lipids in lipid oxidation processes remains contentious (Shewfelt, R.L. 1981). However, FFAs have been found to promote lipid oxidation within frozen stored fish (Lopez-Amaya, C. & Marangoni, A.G. 2000). Physiological lipolysis, associated with hormone sensitive lipase activated by catecholamines and glucagon is intrinsically associated with fatty acid and carbohydrate metabolism. Adrenergic regulation of lipid mobilization is evident within higher order vertebrates (Lafontan, M. & Berlan, M. 1993). However teleost do not appear to possess the same adrenergic response, whereby catecholamine-mediated inhibition of lipolysis is observed in numerous species (van den Thillart, G. et al. 2002). This finding suggests that release of FFA in response to harvest stress may not be associated with an earlier onset of lipid oxidation in PM stored WM.

Myoglobin may also present as both an inhibitor, and promoter, of lipid oxidation in post mortem stored muscle tissue. The thaw process, associated with a rapid drop in pH, and cellular decompartmentalisation (including protein denaturation and proteolysis), can cause the transformation of myoglobin and other heme pigments to act pro-oxidatively, catalyzing lipid oxidation (Carlsen, C.U. & Skibsted, L.H. 2004). Myoglobin may act as a pro-oxidant in lipid systems by up to four different reaction pathways, all involving the degradation of the heme protein structure and release of simpler iron species that will cleave hydro-peroxides from membrane-associated phospholipids (Carlsen, C.U. et al. 2005). As salmonid WM contains low quantities of myoglobin (Richards, M.P. & Hultin, H.O. 2002; Willmer, P. *et al.* 2005), salmon products with the RM removed would be expected to show lesser propensity to oxidize, compared to WM with RM still attached. However, harvest effects on myoglobin may exist that will have an effect on lipid oxidation in the WM. In the period immediately post harvest, potential degradation of myoglobin or hemoglobin, and associated release of iron species may leak into WM aided by the perfusion of blood that can continue post mortem. Also, potentially, under physiological conditions of ischemia, deoxymyoglobin may, under the right conditions, show pro-oxidant activity (Carlsen, C.U. *et al.* 2005). Such physiological conditions may be relevant *in vivo* during escape behaviour elicited during the harvest procedure or within post mortem, anaerobically fuelled, rested muscle. The significance of myoglobin induced lipid oxidation is yet to be empirically determined. If myoglobin was shown to have an effect on lipid oxidation rates of WM, valuable information on the effect of peri-mortem fatigue and post mortem cellular activity could then be ascertained.

Protein Denaturation

During refrigerated storage, a significant decrease in myofibrillar solubility within a high concentration salt solution was observed. There were no significant differences observed in tissue harvested by the two different protocols. Significant decreases in the salt-soluble protein content, and associated decreases in textural strength, have been identified during freeze chilling, and following thawing from the frozen storage of fish tissue. Within Atlantic cod exposed to numerous freeze thaw cycles (-20°C to +20°C) a 15% decrease in the salt-soluble protein content was observed after a single freeze/thaw event, with significant increases observed following each repeat freeze-

thaw event (Benjakul, S. & Bauer, F. 2000). It was concluded that the formation of disulphide bonds between proteins, and hydrophobic interactions resulting from the freeze/thaw process, caused the loss in protein solubility.

Analysis of frozen/thawed cutlets of Atlantic salmon (12 months at -25°C/+4°C), harvested by either CO_2 or two step isoeugenol (AQUI-S®) anaesthesia, identified that fish harvested by the two step AQUI-S® treatment possessed a significantly firmer texture (as measured by a shear test and blunt compression test), than fillets harvested using the CO_2 treatment (Kiessling, A. *et al.* 2004).

Within frozen, pre-rigor chicken breast and leg muscle, a significant shortening of sarcomere length was observed upon thawing at +18°C and 0°C (Yu, L.H. et al. 2005). This shortening had a noticeable effect on textural strength (as measured by a shear test) in the +18°C thaw experiment. Experimental evidence of thaw-shortening was not identified in the pre-rigor/rested thawed muscle tissue. However, personal observations, as mentioned earlier, indicated that tissue from the rested treatment had an apparently greater textural strength, which was more resilient to slicing with a scalpel blade, and held its shape better than tissue from the exercised treatment, upon handling.

CHAPTER 5

The Effect of Frozen and Subsequent Storage at -1°C on Chinook Salmon (*Oncorhynchus tshawytscha*) White Muscle

Abstract

Previous results indicate that, as rested tissue undergoes a thaw event, metabolic activity occurs prior to or at -1°C. Additionally, tissue harvested by rested means demonstrates retarded development of lipid oxidation products when compared to tissue that has undergone vigorous activity when harvested. The current experimental design probes metabolic and lipid oxidation processes that occur in salmon exposed to a limited period of frozen storage before thawing to -1°C. Within rested tissue frozen pre-rigor, abrupt cellular activity identified by the depletion of ATP and glycogen coincident with a gradual increase in lactate, was found to occur between the temperatures -3°C and -1.5°C. Rested tissue was again identified to undergo delayed lipid oxidation processes when compared to exercised tissue, during storage at -1°C. Interestingly, within rested tissue exposed to a six or 24 hour post-mortem chilled storage, lipid oxidation processes were further delayed when compared to rested tissue that was filleted and frozen immediately post harvest.

Introduction

As identified within previous chapters, lipid oxidation processes of exercised and rested tissue occur at similar rates during frozen (-19°C and -9°C) storage. However, upon transfer of tissue from frozen (-19°C) to refrigerated (+4°C) storage, lipid oxidation processes are delayed in rested tissue when compared to exercised tissue. Within this chapter, the effects of storage on rested and exercised tissue exposed to a freeze event, followed by an extended period of storage at -1°C, will be described. As -1°C storage

provides an intermediate temperature slightly above the phase change boundary between the frozen and thawed state it would be expected that low rates of degradation would occur compared to elevated temperatures, prolonging the storage life of the tissue.

Storage of fish at temperatures around the freezing point of muscle tissue is widely practiced within the fish capture and processing industry and is generally acknowledged as a beneficial storage temperature that delays quality deterioration processes (Bramsnaes, F. 1965; Huss, H.H. 1995). Such storage conditions can be attained by numerous methods, including addition of ice to fresh or salt water to form a slurry at 0°C and -0.05°C respectively (Bramsnaes, F. 1965). These methods bring the core temperature of fish tissue down to temperatures just above the freezing point of the muscle. They are commonly practiced on-board fishing vessels and during transport of fish from culture sites to processing facilities. Alternatively, fish can be stored below their freezing point using superchilled storage techniques. Superchilled storage, also referred to as "partial freezing", involves the storage of fish tissue 1-2°C below their freezing point, converting approximately 20-50% of cellular water into ice (Gallart-Jornet, L. et al. 2007). Such temperatures can be achieved by refrigerated means, and are more commonly observed for the storage of fresh fish in vessels undergoing long-duration fishing trips and within processing facilities (Waterman, J.J. & Taylor, D.H. 2001).

In investigating the effect of post mortem storage, I describe tissue undergoing a limited period of frozen storage followed by -1°C storage. Harvest treatments incorporate (i) rested tissue filleted immediately after harvesting and exsanguination, (ii) exercised tissue filleted immediately (as above), (iii) rested tissue exposed to 6 hours post-mortem (PM) storage within chilled water (CW at +5°C) and (iv) rested tissue exposed to 24 hours PM storage in CW. These post mortem storage protocols were incorporated to represent commercial practices. Whereby, harvested fish are often transported from the harvest site to the processing facility. Within rested tissue the ability of rested tissue to continue metabolic processes, post mortem, can be greatly extended (Black, S.E. 2002; Robb, D. 2002). However, as time proceeds post harvest, the rested tissue will continue

metabolic processes, resulting in a gradual decrease in metabolic energy stores and a coincident increase in the byproducts of anaerobic metabolism (Black, S.E. 2002; Robb, D. 2002). Thus, rested tissue that has undergone varying lengths of PM chilled storage will at the time of filleting result in a product in various metabolic states.

A post-mortem storage temperature of +5°C was selected as it was a temperature close to half the acclimated temperature of the fish, which has been identified as a preferable storage temperature for pre-rigor stored Chinook salmon WM (Jerrett, A.R. *et al.* 2000). Dual aims of this experiment are to identify the effect of rested and exercised harvest protocols on lipid oxidation processes within tissue exposed to frozen then -1°C storage, and to identify whether a period of PM storage of rested tissue, prior to filleting and tissue processing, has any effect on lipid oxidation processes in the tissue during storage. Findings will provide valuable information with potential application in industry.

Additional to the investigation of lipid oxidation processes, metabolic processes will be further examined during the thawing of tissue from exercised and rested treatments – filleted immediately post-harvest. As discussed in Chapter 4, upon thawing of frozen tissue to +4°C, metabolic activity occurred prior to, or at -1°C**.** By profiling metabolic activity during the thawing of frozen tissue to -1°C, we will be able to further elucidate the temperature range that these metabolic changes occur.

Materials and Methods

Experimental Fish

Groups of experimental fish were again collected from Isaac Salmon Farm, Christchurch, New Zealand. As described in Chapter 2, fish were transferred from the commercial raceways to the secondary containment facility before experimental harvests were conducted seven (and nine) days post transfer. Two different harvests protocols were conducted (exercised and rested), where rested tissue was exposed to various lengths of PM storage. Ultimately, tissue in different states of peri-mortem fatigue or PM metabolic run-down was presented.

Harvest and Fish Handling Procedures

All fish harvest procedures were conducted with the approval of the University of Canterbury Animal Ethics Committee, Approvals 2006/8R and 2006/24R. Fish were harvested using 'rested' and 'exercised' methodologies, performed as described in Chapter 2. Within the first harvest procedure (October), two groups of fish were harvested by either rested (n=7) or exercised (n=7) means before being transported to the university and tissue preparation steps carried out. These fish representing the rested and exercised treatments were filleted, sampled and frozen immediately. For the treatment involving the PM storage of whole fish in CW, one group of fish (n=7) was harvested (in November) by the rested methodology. The fish were then transferred from the harvest site within three 40L chilli-bins, containing chilled water (6°C, chilled with ice) while being allowed to bleed-out. Once fish had been transported, they were transferred to a 56L plastic fish-bin containing chilled water (CW) held at 5°C within a constant temperature room. Following a six hour period of PM storage, the fish were removed from chilled storage, and a fillet removed, from which muscle blocks were excised then freeze-clamped. The carcass was then placed within a water-tight plastic bag and returned to the refrigerated water. 24 hours post harvest, the remaining fillet was taken from the fish and WM tissue blocks excised before freeze-clamping. All procedures not detailed, including euthanisation, exsanguination, and tissue preparation were performed as in Chapter 2.

Tissue Storage

Following the freeze clamp procedures performed for all four treatments, tissue blocks were labelled and wrapped in aluminium foil, before a block of muscle from each of the fish within a treatment was randomly selected and placed collectively in small, unsealed plastic bags labelled by harvest (and PM storage) treatment. For fish from the October harvest treatment, exposed to a freeze/thaw event, a single slice of tissue was freeze-clamped with a t-type thermocouple wire embedded within its core, before being wrapped with aluminium foil. Within the unsealed sealed plastic bag containing the tissue slice with thermocouple wire embedded, additional pieces of tissue were added so as to standardise the number of tissue blocks contained.

Within the October harvest group, fish collected by rested means on the first of the two harvest days (i.e. seven days post transfer) were held at -80°C until fish sampled at the latter date (exercised fish harvested nine days post transfer) were added. Tissue samples from both treatments (and tissue used to profile temperature) were held at -80°C for a further 24 hours before transfer to the -1°C storage receptacle. For the rested group harvested in November, tissue that was excised and freeze-clamped six hours PM was held at -80°C until the remaining samples were excised and freeze-clamped 24 hours post harvest. Both sets of tissue were held at -80°C a further 24 hours before transfer to -1°C. Temperature control at -1°C was provided by the EvaKool/Tropicool Thermoelectric unit.

Sub-sampling of Tissue

During thawing, sampling of the rested and exercised tissue – filleted immediately was performed at selected temperatures during the warming event. Using the tissue slice with embedded thermocouple wire as a model, samples of tissue were removed coinciding with selected temperature points. After removal from the storage receptacle, the wrapped pieces of tissue were freeze-clamped under liquid nitrogen, then transferred to -80°C before analysis.

During profiling of secondary lipid oxidation products, sampling of tissue occurred at time zero (i.e. upon freeze-clamping) and three day intervals following thereafter. After removal from the storage receptacle, the wrapped pieces of tissue were freeze-clamped under liquid nitrogen, then transferred to -80°C before analysis.

Analytical Methods

Analytical methods used within this experimental set can be seen in Chapter 2. The assay kits used to determine metabolite concentrations are listed below.

Glucose Analysis: Gluco-quant Glucose/HK Assay kit (Cat # 11447521 216), Roche, Mannheim, Germany)

Lactate Assay: Megazyme Lactic Acid Kit (Catalogue number K-Late)

ATP Assay: Enliten® (Cat#FF2021, Promega Corporation, WI, USA)

Temperature Profiling

Temperature profiles within the cold storage units were logged using the HOBOPro internal temperature loggers, placed within the container holding the tissue samples. Tissue temperatures were logged using the PowerLab t-type thermocouple setup, with the thermocouple lead embedded within a single tissue slice.

Statistical Analysis

Comparison of the post harvest condition of the fish harvested using the two different harvest techniques was performed using a Student's t-test. Analysis of oxidative degradation profiles from tissue from the four different treatments used a Two Factor ANOVA (repeated measures) with a Bonferroni Post-test. This statistical analysis enabled comparison between treatments and identification of significant changes in the data over time. For samples from the rested – filleted immediately treatment, missing values from the experiment (as n=6 rather than n=7) were replaced with mean values from each sample period within the treatment, thus allowing comparison using Two Factor ANOVA.

Results

Post Harvest Fish Condition

The condition of post harvest fish utilised in the experimental set presented is outlined below (Table 5-1).

Table 5- 1: Morphological and Physiological State of Experimental Fish at the Time of Harvest. (results expressed as mean ± sd. Significant differences, as compared to the rested treatment from the October harvest, are denoted (*p<0.05, **p<0.01, ***p<0.001).

	October Harvest		***November Harvest***
	Exercised	Rested – Filleted immediately	Rested – 6 & 24 hours chilled storage
Sample Size (N)	7	6	7
Plasma Lactate (mM)	5.26 ± 0.99 ***	2.78 ± 0.60	1.48 ± 0.50***
Plasma Glucose (mM)	3.54 ± 0.73 **	2.30 ± 0.56	1.13± 0.32 ***
Weight (g)	2853.2 ± 666.7	2345.7 ± 485.3	3143.57 ± 623.97 ns
Fork Length (mm)	54.1 ± 3.2 ns	53.9 ± 6.3	59.29 ± 3.50*
Condition Factor	1.67 ± 0.38	1.51 ± 0.23	1.50 ± 0.20 ns
GSI	0.68 ± 0.08 ns	0.70 ± 0.31	0.92 ± 0.08 ns
HSI	0.87 ± 0.31 ns	0.91 ± 0.12	0.92± 0.08 ns
Sex	XX (7/7)	XX (6/6)	XX (7/7)

Within the rested treatment of the October harvest group, one fish was excluded from further analysis. The fish excluded was of poor condition with a broken spinal column and pathological symptoms such as pale flesh, swim bladder oedema, low liver weight and undeveloped gonads. Sample size of the rested treatment group, October harvest, was reduced to six.

From the October harvest group, significant differences existed in plasma glucose and lactate concentrations. This indicates that the fish were harvested in significantly different physiological states, with exercised fish experiencing higher levels of anaerobic activity bought on by the harvest protocol.

Comparing rested treatment groups from the October and November harvests, significant differences can be seen in plasma lactate and glucose concentrations. This suggests that fish from the November harvest were more rested, either within the harvest treatment or prior to harvesting. The fish from the November harvest were insignificantly larger in mass, and significantly longer. Morphometric indices were not significantly different.

Fillet Condition

The condition of fillets removed from fish, prior to WM blocks being excised are presented below (Table 5-2).

Table 5- 2: Physiological State of Experimental Fish at the time of Filleting.

(results expressed as mean ± sd. Significant differences, as compared to the rested treatment from the October harvest, are denoted (*p<0.05, **p<0.01, ***p<0.001).

	October Harvest		*November Harvest*	
	Exercised	**Rested – Filleted immediately**	**Rested – 6 hours chilled storage**	**Rested – 24 hours chilled storage**
Cut Surface pH	6.54 ± 0.04 ***	7.58 ± 0.08	7.38 ± 0.08***	6.5 ± 0.1***
Tissue ATP ($\mu mol.g^{-1}$)	0.7 ± 0.1***	6.7 ± 1.3	5.7 ± 0.7	0.4 ± 0.7***
Tissue Glucose ($\mu mol.g^{-1}$)	3.9 ± 1.1 ^{ns}	2.8 ± 0.7	3.9 ± 1.1 *	3.2 ± 0.6 ^{ns}
Tissue Glycogen ($\mu mol.g^{-1}$)	2.2 ± 1.9***	13.6 ± 3.3	14.2 ± 3.2	3.2 ± 2.1***
Tissue Lactate ($\mu mol.g^{-1}$)	27.7 ± 3.8***	8.2 ± 0.5	11.69 ± 2.95**	34.12 ± 8.47***
Rigor Stage	Early stages of rigor onset	Pre-rigor	Pre-rigor	Full-rigor

Cut surface pH values were all significantly different when compared to fish from the rested treatment, filleted immediately. Low pH values were identified in exhausted and 24 hour CW stored tissue. These values were comparable, identifying peri-mortem fatigue/metabolic rundown post-mortem. Tissue levels of ATP, lactate and glycogen were not significantly different in rested tissue filleted immediately and fish stored in CW for 6 hours. This indicates the ability of rested tissue to continue metabolic processes and maintain cellular viability, post-mortem. Significant differences in tissue ATP and glycogen concentrations were seen in fish harvested by exercised methods and in tissue stored 24 hours in CW. Within these treatments, stores of ATP and glycogen had depleted to undetectable levels, while a significant increase in tissue lactate had occurred, collectively identifying the depletion of metabolic energy stores.

Associated with differences in metabolic energy stores and cut surface pH levels, differences in the rigor state of fish from the different treatments were also observed. Those fish within, or entering, rigor had low tissue concentrations of ATP and glycogen, coincident with high lactate concentrations. This indicates that PM metabolism had ceased and tissue could no longer respire.

Storage Temperatures

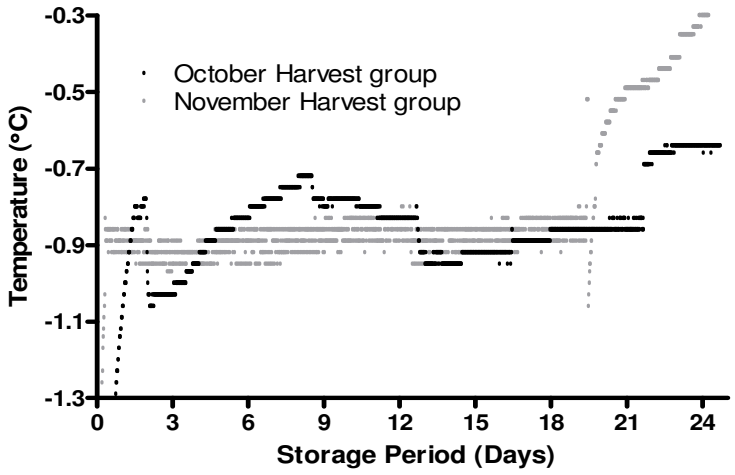

Figure 5- 1: Temperature Profile of -1°C Storage Unit

Within this experiment, tissue treatments were exposed to a freeze event and -1°C storage at different points in time with the second group of fish entering -1°C storage following the end of the first group's storage period. The first group, harvested in October, included the rested and exercised tissue filleted immediately, while the second group, harvested in November, consisted of the rested tissue that underwent PM storage within CW. Storage temperatures of the two separate -1°C storage experiments were found to be comparable between experiments. The mean temperature, during the storage of exercised and rested tissue – filleted immediately was -0.8±0.2°C (mean±sd) and -0.9±0.1°C (mean±sd) in tissue stored PM in CW. Within the CW storage experiment, a large elevation of the storage temperature above ≈-1°C was seen after 18 days. For this reason, statistical comparison of analysis values after 18 days of storage between the two different experiments were not performed.

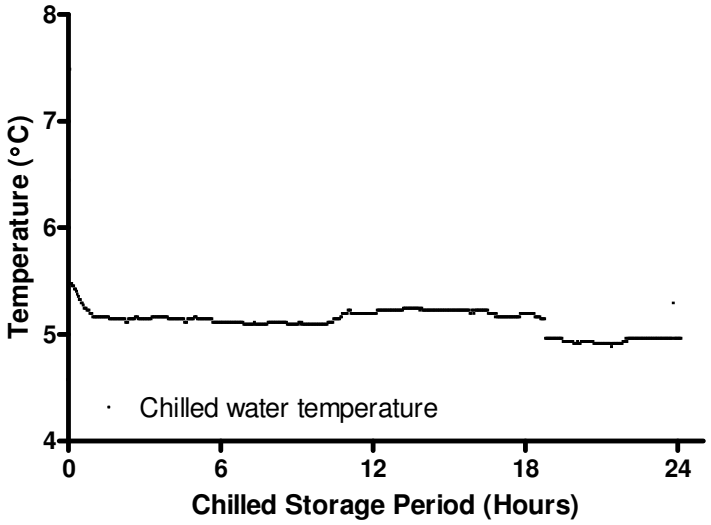

Figure 5- 2: Temperature of CW during Post-harvest Storage

Upon transfer of newly harvested, transported fish into CW, a small increase in temperature was seen owing to the transfer of residual heat from fish. Temperatures equilibrated to ≈5°C within one hour at controlled temperatures. The mean temperature of the RW during the post harvest storage period was 5.0±0.2°C (mean±sd).

Temperature Profile During Thawing

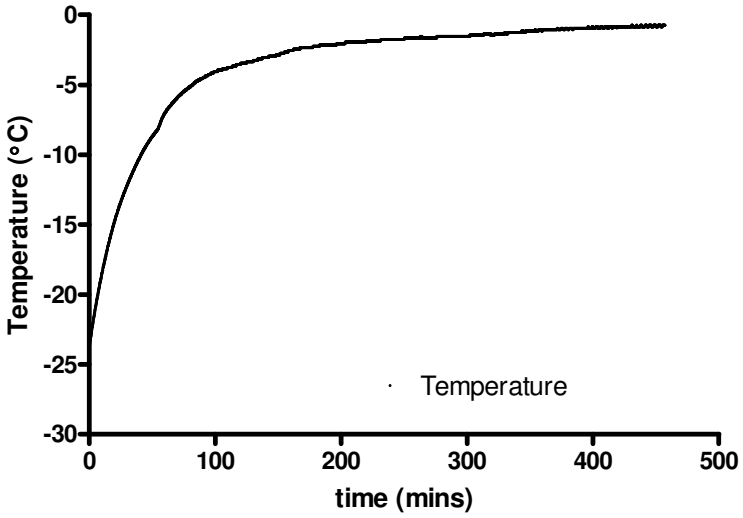

Figure 5- 3: Core Temperature of Tissue Slices upon Thawing

Blocks of tissue thawed to -1°C demonstrated a much more gradual (459 minute) equilibration to the ambient temperature (-1°C) than tissue warmed to +4°C which reached -1°C after 85 minutes (and equilibrated to +4°C after 107 minutes). Identifying the thaw temperature and latent heat of fusion zone is difficult owing to the slow rates of change observed. The time point that demonstrates the enthalpy of fusion appears to be around 300 minutes, coinciding with a temperature of -1.3°C.

Thaw Metabolism

Table 5- 3: Tissue ATP Concentrations during Thawing to -1°C.

(Key: *p<0.05, **p<0.01, ***p<0.001. Data is expressed as mean ± sd)

	Time Elapsed (min)	Tissue ATP ($\mu mol.g^{-1}$) - rested	Tissue ATP ($\mu mol.g^{-1}$) - exercised
	0	6.7 ± 1.3	0.6 ± 0.8
-5°C	88	7.3 ± 2.0	0.1 ± 0.1
-3°C	146	6.2 ± 1.5	0.4 ± 0.5
-1.5°C	320	0.0 ± 0.0***	0.0 ± 0.0
-0.9°C	1188	0.0 ± 0.0***	0.0 ± 0.0

Table 5- 4: Tissue Lactate Concentration during the Thaw Event.

(Key: *p<0.05, **p<0.01, ***p<0.001. Data is expressed as mean ± sd)

Temperature	Time Elapsed (min)	Tissue Lactate ($\mu mol.g^{-1}$) - rested	Tissue Lactate ($\mu mol.g^{-1}$) - exercised
	0	8.2 ± 0.5	27.7 ± 3.8
-5°C	88	6.1 ± 1.4	29.2 ± 2.7
-3°C	146	11.6 ± 6.3	36.0 ± 11.3*
-1.5°C	320	37.2 ± 10.2 ***	35.3 ± 6.7*
-0.9°C	1188	34.1 ± 4.5***	38.5 ± 4.9*

Table 5- 5: Tissue Glycogen Concentration during the Thaw Event.

(Key: *p<0.05, **p<0.01, ***p<0.001. Data is expressed as mean ± sd)

Temperature	Time Elapsed (min)	Tissue Glycogen ($\mu mol.g^{-1}$) - rested	Tissue Glycogen ($\mu mol.g^{-1}$) - exercised
	0	13.6± 3.3	2.2 ± 1.9
-5°C	88	10.7 ± 4.2	0.8 ± 0.8
-3°C	146	10.7 ± 3.3	1.0 ± 0.5
-1.5°C	320	0.9 ± 0.4***	1.1 ± 0.9
-0.9°C	1188	0.8 ± 0.9***	2.2 ± 1.9

Tissue concentrations of ATP, lactate and glycogen showed the typical significant differences associated with the rested and exercised harvest techniques, with stores of ATP and glycogen being significantly higher ($p<0.001$) and lactate being significantly ($p<0.001$) lower within rested tissue at time zero.

During the warming of rested tissue transferred to -1°C, no significant changes in the concentration of lactate, glycogen or ATP were observed until the tissue had reached -1.5°C, identifying that metabolic activity occurs between -3°C and -1.5°C. Highly significant ($p<0.001$) changes in tissue ATP, lactate and glycogen were observed in rested tissue, while a significant change ($p<0.05$) was observed in the tissue lactate concentrations of exercised tissue at -3°C. There were no significant changes in the level of ATP and glycogen within exercised muscle.

Lipid Oxidation

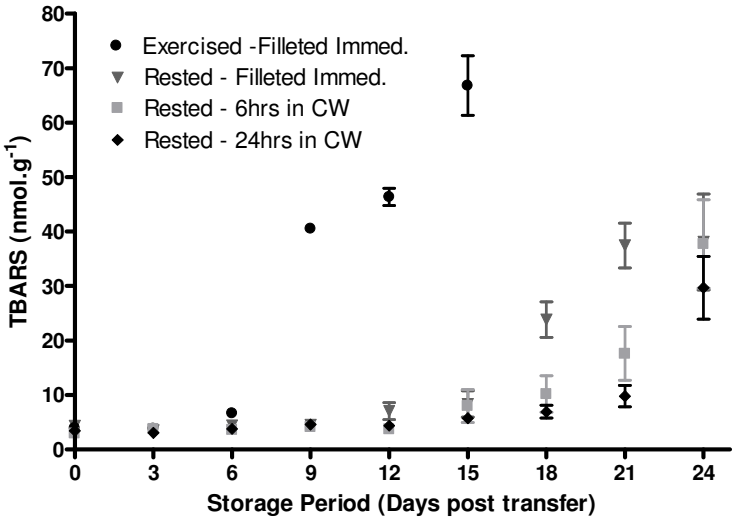

Figure 5- 4: Lipid Oxidation Profile in WM Tissue Blocks following Transfer to -1°C (data plotted as mean±s.e.m)

Analysis of lipid oxidation product development performed using two factor ANOVA identified a significant effect associated with time ($p<0.001$) and treatment ($p<0.001$). Analysis by Bonferroni post-test identified a significant increase in lipid oxidation products (when compared to time zero values) at nine days storage ($p<0.001$) within exercised tissue – filleted immediately, at 18 days storage ($p<0.001$) within rested tissue – filleted immediately, at 21 days storage ($p<0.001$) in rested tissue exposed to six hours PM storage, and 24 days in rested tissue held for 24 hours in CW ($p<0.001$). Note, storage period is measured as days post transfer from -80°C, not days post harvest.

Statistical analysis by Two Factor ANOVA – repeated measures identified that differences between the exercised and rested tissue – filleted immediately treatments were highly significant ($p<0.001$). Comparison of six hour, and 24 hour CW storage treatments identify that differences between treatments were not statistically different ($p=0.083$). Differences between rested fillets – filleted immediately, and rested fillets exposed to six and 24 hours CW storage were significantly different ($p<0.001$ in both instances).

Discussion

Methods

Comparison of fish from different harvest groups, harvested at different points in time has associated drawbacks. The experimental design necessitated that three harvests be performed, thus allowing comparison of different harvest (and post-harvest storage) treatments. Attempts were made to minimize variation between test fish, with fish being collected from the same raceway at Isaac fish farm. Variation was also minimised by utilising one fillet each from fish within a single rested harvest event for each of the six hour and 24 hour PM storage experiments. Using this methodology, the tissue of each fish within the post-mortem storage experiment profiled was affected only by one of two different post-mortem storage treatments, and not by differences associated with the harvest event or life-history.

Issues arose during PM storage of the fish in CW, as following the removal of one fillet, the carcass was required to be placed back into chilled water. To minimise osmotic effects associated with exposure of cut muscle tissue to fresh water, the carcass was sealed in a large plastic bag. This however allowed limited contact between the cut muscle surface and atmospheric oxygen. As the exposed surface remained on the opposite side of the spine to that of the second fillet that was removed, a layer of tissue acted to serve as a buffer zone, preventing permeation of oxygen through to the d-block muscle that was later excised 24 hours post harvest.

Fish and Tissue Condition

Fish from the October rested harvest group had moderately high concentrations of lactate and glucose within the plasma component of the blood. This suggests that fish from the October harvest may have suffered increased levels of stress or elevated levels of activity prior to, or during the harvest event. During early October, the secondary containment facility was experiencing intermittent periods of low water flow, possibly causing the fish to survive in a hypoxic environment for limited periods. Such a situation occurring prior

to the harvest event in question, would support the assumption that the fish had experienced prior levels of stress/activity.

Rested tissue, filleted immediately, possessed high levels of ATP and glycogen, with low levels of lactate. Cut surface pH values were high, supporting the fact that the tissue had been harvested in a rested manner. Conversely, exercised tissue held low concentrations of ATP and glycogen and high concentrations of tissue lactate. Fish held within chilled storage, PM, showed significantly lower cut surface pH levels than rested tissue filleted immediately. Tissue lactate concentrations increased within the storage period, while tissue ATP and glycogen stores were depleted by 24 hours storage. Insignificant differences between tissue ATP and glycogen stores within rested tissue filleted immediately and rested tissue stored for six hours post-harvest, suggest that, prior to harvesting, fish from the November harvest group possessed higher glycogen, and potentially higher ATP reserves than fish harvested in October. Tissue stored for 24 hours post-mortem was in a comparable metabolic state to exercised tissue, with depleted levels of ATP, glyocogen and high levels of lactate. Ultimate cut surface pH values of these two treatments were directly comparable.

Characteristics of the rested, exercised and post-mortem stored muscle were comparable to fish harvested using similar protocols, within the literature. Post-mortem storage of trout harvested using rested protocols (incorporating AQUI-S®), identified a cut surface pH value of 7.7 immediately post harvest. This pH value had decreased to ≈7.3 after 10 hours storage, presumably on ice. The ultimate pH value of 6.5 was not reached until 40 hours post harvest (Robb, D. & Warriss, P. 1997). Similar results were reported in salmon (*Salmo salar*) muscle during post-mortem storage. Fish harvested via a rested protocol (again utilizing AQUI-S®) had a cut surface ph of 7.2 ≈8 hours following slaughter: this value dropped to an ultimate pH of 6.3 between 35-40 hours after harvest (Robb, D. & Warriss, P. 1997). Salmon harvested with AQUI-S® did not enter rigor until 24 hours after harvesting, whereas fish harvested by protocols that induced high levels of activity within the fish entered rigor as early as four hours following harvest (Robb, D. & Warriss, P. 1997). Differences associated with the ultimate pH attained and rates of rigor

onset occur, but are likely related to differences between species, life histories and handling protocols. Post-mortem storage of rested Chinook salmon observed within this experiment is comparable to numerous other results within the literature that detail post-harvest metabolism and the onset of rigor-mortis within salmonids (Azam, K. *et al.* 1989; Sigholt, T. et al. 1997; Jerrett, A.R. *et al.* 2000; Robb, D. 2002; Roth, B. *et al.* 2006).

Cellular activity During Thawing to -1°C

During thawing of tissue to -1°C, metabolic activity was demonstrated as in Chapter 4. This metabolic activity, marked by a decrease in cellular ATP, glycogen and an increase in lactate, occurred between -3°C and -1.5°C as identified by the significant changes that occur at the 1.5°C sampling temperature. These results confirm the results of Chapter 4, and provide additional information by identifying the narrow temperature band over which cellular activity occurs.

In Table 3-1, the proportion of free water within cod muscle stored at temperatures between -1°C and -20°C was outlined. Between -3°C and -1°C there is a rapidly decreasing volume of ice-crystals, from 66% to 8%, within the tissue (James, S.J. 2000). This zone of rapid de-crystallisation coincided with the temperature zone in which cellular activity was demonstrated in the above experiment. This indicates that cellular activity in thawing tissue is tightly related to the volume of water in the un-frozen phase.

During glycolytic, anaerobic metabolism each molecular unit of glucose is broken down into two molecular units of lactate. Within this experiment, time zero glycogen stores of rested tissue were ≈14µmol.g^{-1}, depleting to zero following thawing, while tissue lactate concentrations were ≈8µmol.g^{-1}, increasing to 34.5µmol.g^{-1}, post transfer. Applying the ratio presented to the observed results, 14 molar units of glucose (glycosyl units) produce 26µmol.g^{-1} of lactate, grossly accounting for the observed increase in tissue lactate. These calculations identify that during thawing, anaerobic glycolysis was able to occur in typical fashion.

Glycolysis during the thaw event was discussed within Chapter 4. Further information associated with the temperature at which these glycolytic/thaw rigor processes occur has been demonstrated within carp, red sea-bream and Atlantic cod muscle tissue (Ma, L.B. & Yamanaka, H. 1991; Ma, L.B. et al. 1992; Cappeln, G. *et al.* 1999). For Red sea-bream frozen pre-rigor, then thawed under running tap water (+18°C), a depletion in tissue ATP coincident with an increase in lactate and inosine-monophosphate concentrations was observed at -1.6°C. The same pattern of ATP depletion and lactate/IMP accumulation was demonstrated within pre-rigor frozen carp thawed under the same protocol. Cellular activity was demonstrated to occur at -1.1°C within this species (Ma, L.B. & Yamanaka, H. 1991). The reported rupture of the sarcoplasmic reticulum during freeze/thawing and resultant Ca^{2+} leakage was suggested as the reason for abrupt ATP consumption, probably owing to the activation of myofibrillar Mg^{2+} ATPase (Ma, L.B. *et al.* 1992). Within pre-rigor frozen Atlantic cod, maximum depletion of high energy phosphorous compounds was observed to occur around -2°C (compared to -5°C, -6°C and -10°C), during thawing to 0°C (Cappeln, G. *et al.* 1999).

When the cellular activity of thawing Chinook salmon muscle is compared to other species, it can be seen that rapid depletion of ATP, coinciding with a rapid decrease in glycogen and increase in tissue lactate, will occur when fish tissue is frozen and thawed in a pre-rigor state. Assuming temperature measurements within the cited literature are accurate, slight differences in the temperature at which cellular activity occurs upon thawing may be observed between species. Potentially, these differences may relate to whether the fish are acclimated to fresh-water or marine environments, as tissue osmolarity (which is lower in fresh-water species) and water content may have an effect on freeze and thaw properties.

Development of Lipid Oxidation Products associated with Harvest Treatment and Post Mortem Chilled Storage.

As seen previously (Chapter 4), rested tissue demonstrates delayed development of lipid oxidation products, when compared to exercised tissue, during chilled storage. Following a freeze/thaw event, rested tissue stored at -1°C did not show a significant increase in

lipid-oxidation products until 18 days storage, whereas exercised tissue showed a significant increase after only nine days storage, half that of rested tissue. Surprisingly, further retardation of lipid oxidation processes were demonstrated in rested tissue that had been exposed to a PM chilled (+5°C) storage period following harvest. These differences associated with the different treatments were confirmed to be statistically significant. Tissue exposed to six hours chilled PM storage (which had showed a significant decrease in cut surface pH values) did not show significant increase until 21 days storage at -1°C, while tissue stored for 24 hours (which had progressed into full rigor) did not show a significant increase until 24 days at -1°C. Differences between these two treatments were not statistically different, although with a p-value of 0.083 they are near significant.

As the rested tissue – filleted immediately, and rested tissue exposed to PM chilled storage (+5°C) were collected from two separate groups of fish, it is possible that variation within the groups of fish used could cause the significant differences observed between treatments and the rates of lipid oxidation product development. A possible cause for the observed difference may relate to the physiological state of the animal peri-mortem, as fish from the first harvest group – filleted immediately showed elevated blood glucose and lactate concentrations when compared to fish from the second harvest group – stored PM in CW. This result potentially lends support to the hypothesis that the rested physiological state retards lipid oxidation processes. However, tissues exposed to six and 24 hour PM chilled storage were collected from the same fish, and thus differences in the rate of onset, and near significant differences in the results can not be explained by inter-fish variation.

Possible mechanisms for retarded lipid oxidation processes in tissue exposed to PM chilled storage may relate to metabolic or cellular events prior to, and/or during the thaw event. Post mortem chilled storage involves catabolism of adenosine tri-phosphate to other purines including ADP, AMP, IMP, Ino, Hx, xanthine and ultimately uric acid. Of these purine catabolites, uric acid has been suggested to possess antioxidant activity within physiological systems (Ames, B.N. et al. 1981), while inosine has been identified

as an effective antioxidant in-vitro (Gudkov, S.V. et al. 2006). However, the rate of accumulation of these two catabolites in rested tissue frozen, and then stored at +5°C is not known. Whether these catabolites posses antioxidant activity in post mortem skeletal muscle/meat is unknown/currently being investigated.

Another possible mechanism for delayed lipid oxidation in fish tissue that underwent a PM storage phase may involve the greater distribution of isoeugneol to cellular compartments. With the depletion of cellular ATP stores, cellular ion pump activity will be affected. Homeostatic control of the cell is lost and the cellular membrane becomes hyper-permeable. Isoeugenol, as it is highly lipophilic, may become more widely associated with membrane bound lipid components such as the cellular membrane, or reduced metal species leaching from the cell.

A further mechanism by which rested tissue, filleted pre-rigor, may be more sensitive to lipid oxidation processes, may relate to the filleting of muscle tissue that is still metabolically active. Whether filleting viable tissue results in oxidative injury to the cellular tissue, either by a depletion of endogenous antioxidants or generation of ROS, caused by exposure of atmospheric air to the exposed surface area of tissue, is speculation. However, it may exist as a potential mechanism that causes the earlier development of secondary lipid oxidation products in rested tissue filleted pre-rigor.

Cellular activity during the thaw is another potential process that may explain differences in lipid oxidation processes in PM stored rested muscle. As rested tissue stored for 24 hours possessed depleted ATP stores and had entered RM, no cellular activity would have occurred during transfer and thawing to -1°C. Other rested tissue groups, which retained high levels of cellular ATP prior to freezing, would have undergone cellular activity upon transfer, which also was associated with a lesser ability to retard lipid oxidation processes. Cryo-preserved viable sperm, of numerous vertebrate species, is known to generate ROS (such as NO and O_2^- (superoxide) radicals) upon thawing (Suvro Chatterjee, C.G. 2001; Kumar, S. & Das, G.K. 2005). The mechanism of this ROS generation within sperm is not well understood (Kumar, S. & Das, G.K. 2005), and to my

knowledge, has not been investigated within viable muscle tissue that undergoes a freeze/thaw event.

Differences in the development of lipid oxidation products between rested fish – filleted immediately, and rested fish stored for six and 24 hours, could potentially relate to the time period that tissue was stored at -80°C, prior to transfer to -1°C storage. Rested tissue – filleted immediately, stored for six hours or stored for 24 hours, was held in frozen storage (-80°C) for 72, 48 and 24 hours respectively. Whether these small differences in the duration of frozen storage (i.e. ice crystal growth or aggregation) had any effect on resulting differences in lipid oxidation is unknown. However, if the ability of rested tissue to retard lipid oxidation was affected by frozen storage, it suggests the mechanism by which rested tissue gains protection is adversely affected by the freezing or thawing process.

Further investigation on the effect of PM chilled storage on the antioxidant properties of rested muscle needs to be investigated before firmer conclusions can be drawn.

CHAPTER 6

General Discussion

The benefits of rested harvesting on finfish quality have been well established in freshly harvested and chilled tissue. These benefits, including elevated textural strength, lower rigor tensions, altered colour scores and reduced tissue gaping, have all been observed when compared to exercised or conventionally harvested tissue (Jerrett, A.R. *et al.* 1996; Robb, D. & Warriss, P. 1997; Jerrett, A.R. & Holland, A.J. 1998; Robb, D. 2002; Kiessling, A. *et al.* 2004; Roth, B. *et al.* 2006). However, few investigations into the effect of rested harvesting procedures on frozen fish quality have been carried out. Previous attempts at improving frozen fish quality have been made by altering the freeze and thaw protocols (Li, B. & Sun, D.-W. 2002; Lakshmanan, R. et al. 2005), enriching grow-out diets with antioxidants (Chaiyapechara, S. et al. 2003), post mortem addition of antioxidants and protective coatings to cut fillets (Pazos, M. et al. 2005; Sathivel, S. 2005) and modified atmosphere packaging (Kristinsson, H.G. et al. 2006).

Within this thesis, the effect of the harvest protocol as a determinant of frozen product quality was investigated. Throughout prolonged frozen storage, rested tissue maintained significantly higher concentrations of soluble protein, but did not show any retardation of lipid oxidation processes. However, once removed from frozen storage and placed at chilled temperatures, rested tissue did demonstrate significant retardation of lipid oxidation. The stability of high energy metabolites during frozen storage was also investigated, as well as changes in these concentrations during the thawing of the tissue.

Critique of Experimental Methodologies

Harvest Protocols

Harvest protocols utilised within this thesis were selected to represent actual and potential commercial harvest practices, with the intention that results obtained could provide a benchmark for commercial operators. Both the rested and exercised protocols were performed on a smaller scale in an environment with greater control of external variables and stressors (i.e. weather conditions, interspecies interactions and peripheral human activity) than commercial operators would face. Despite the advantages inherent in the experimental rested harvest protocols utilised within this thesis, obtaining fish in the same physiological state, within large scale commercial settings is entirely possible, and has been observed (Black, S. et al. 2007)

The exercised harvest procedure utilised, selected to mimic conventional – high stress harvesting practices, was shown to promote escape behaviour within test fish. This activity subsequently presented as a depletion of high energy phosphagen and glycolytic energy reserves. The exercised protocols employed within this thesis could be considered less stressful than those employed within commercial settings. In practice, stresses imposed on fish during harvesting are often more prolonged in time and also more intrusive in nature (personal observation). For this reason, and the fact that not all fish showed complete depletion of ATP and glycogen reserves, fish harvested by the conventional protocol have been termed ‘exercised’ as opposed to ‘exhausted’.

Storage and Core Temperature Profiles

Storage temperatures utilised within this thesis were selected to represent storage temperatures typically used within industry and domestic settings, thus allowing direct application of findings. Consistency of the storage temperature was highly related to the storage unit utilised. The most tightly controlled storage temperature was +4°C (Figure 4-1), owing to the use of a refrigerated water bath. -1°C was the next most tightly controlled temperature (Figure 5-1), and used the thermoelectric storage unit. The frozen storage unit utilised for frozen storage (-9 and -19°C) showed the least tight temperature

control (Figure 3-1). The frozen storage unit utilised conventional compression cycle means to achieve the temperature. There were no defrost cycles incorporated in the run program of the storage unit. Variation in the storage temperatures most probably related to the thermostat mechanism associated with each storage device. The Lauda immersion thermostat, used for temperature control of the +4℃ water bath, was the most advanced temperature control unit, incorporating a proportional temperature control algorithm. Additionally, temperature control of a water bath at +4℃ had the inherent benefit of maintaining a constant temperature of a thermal mass with a high heat capacity, as opposed to maintaining a constant temperature within a medium of air, as performed at all other storage temperatures. The Tropicool™ thermostat unit maintained an acceptably constant temperature profile throughout -1℃ storage. The tropicool unit incorporates a basic on/off analogue thermostat to maintain a constant temperature, its positioning within the cooled air flow allows a fast response time and tight temperature control (R Stuart, Tropicool Ltd, personal communication, 26 February 2008). The Waeco CoolFreeze frozen storage unit also had a simple on/off thermistor type thermostat (D Newman, Waeco Australia, personal communication, 29 February 2008). Simplicity of the control circuit, or the utilisation of an undersized thermistor/sensor could be responsible for the poorer temperature control characteristics of the Waeco storage unit (when compared to the other two temperature control units).

Recording of storage temperatures and tissue core temperature provided profiles of the storage environment the WM tissue blocks were exposed to during storage. These acted as a cross-check that would identify any deviations from the set storage temperature and potentially explain any unusual results that could arise. Storage temperatures remained acceptably constant throughout all storage periods (with the exception of prolonged -1℃ storage).

Recording the core temperature of muscle blocks entered, then removed from frozen storage provided an indication of the rate of thawing, and temperature/state transitions of the tissue. Thawing of tissue blocks occurs in a directional gradient from the outside in (Golden, D.A. & Arroyo-Gallyoun, L. 1997). Placement of the thermocouple lead into

the core of the tissue block was the most practical arrangement. The geometry of the tissue blocks (of approximate size 20x30x5mm) required that the thermocouple lead was placed at mid depth, at the centre of the square, i.e. the centre-most point of the tissue assuming a constant cross sectional area of the tissue block.

Calibration of thermometers (including thermocouples and RTDs) was performed via a two point calibration in iced (0°C) and tap water (≈18°C). Temperatures were cross-referenced to a red bulb thermometer and digital temperature probe (Checktemp, Hanna Instruments, Melbourne, Australia). Calibration was repeated until verified measurements of three different temperatures had been recorded. Incorrect calibration of the t-type thermocouple unit had the potential to inaccurately report temperature events such as the zone of latent heat of fusion. As two different thaw experiments reported the same temperature at which this event occurred, and the results agreed with other published values of freezing temperature of Chinook salmon WM (Jerrett, A.R. *et al.* 2000), the results acquired using the t-type thermocouple were likely to be accurate.

Storage Conditions

During storage, tissue blocks were placed in unsealed plastic bags (or in the case of +4°C unsealed Falcon tubes) throughout the duration of storage. The mass of tissue in each bag/tube was crudely standardised by putting equal numbers of tissue blocks into each receptacle. A more thorough standardisation of tissue mass per storage receptacle could be have been achieved by placing equal numbers of standard weight tissue blocks into a fixed volume (and potentially fixed composition) of gas/air. This would have allowed tighter control of potential variation in storage conditions.

Lipid Oxidation Processes:

The TBARS assay is the most frequently utilised measure of lipid oxidation in biological systems (Ross, C.F. & Smith, D.M. 2006). TBARS measurements are based on the formation of a TBA adduct with some of the aldehydic lipid oxidation products. MDA is one of these potential products and is used as the reference standard. Other products from the oxidation of lipids, not measured by the TBARS assay, include lipid hydro-peroxides, conjugated dienes, and numerous other low molecular weight alkanes, aldehydes and

ketones (Dotan, Y. et al. 2004). Thus, measurement of aldehydes structurally related to MDA do not present an all encompassing determination of lipid oxidation processes occurring during storage of Chinook salmon WM tissue.

Aluminium foil was used to wrap muscle blocks throughout the duration of frozen storage. Aluminium foil presents a highly impermeable barrier to moisture and oxygen (Lamberti, M. & Escher, F. 2007), however, as used within this investigation, the wrapped tissue blocks should not be considered airtight. Also, potential interactions between the lipid component of the muscle block and the aluminium oxide layer could have an effect on the development of lipid oxidation products within the stored tissue. Previous literature has investigated the effect of aluminium foil and polymeric cling film on biogenic amine formation and sensory determination of sea bream (*Sparus auratus*) during chilled (+2°C) storage, with minimal differences between the two different materials observed (Kuley, E. et al. 2005; Ozogul, F. et al. 2007). However, specific investigations into lipid oxidation processes, as affected by an aluminium foil wrap, during chilled and or frozen storage, have not been performed.

Protein Solubility Assay:

Measurements of protein solubility in a high concentration salt solution proved problematic throughout the duration of the investigation. Within each individual sampling period, high levels of variation were observed, despite analyses in duplicate, as evidenced by the large error bars (representing s.e.m.) on Figures 3-9 and 4-10. Variation in protein solubility values may have been associated with variation in the tissue (i.e. variable levels of denaturation within a tissue block or the potential inclusion of collagen within myofibrillar tissue weighed for analysis). However, one would expect the process of grinding under mortar and pestle to disguise this variation within an individual tissue slice. Utilisation of the BCA protein determination/assay kit could have been the source of the variation as numerous substances are known to interfere with determinative absorbance readings (Pierce 2005b). Avoidance of lipid interactions was performed by the addition of Triton-X to the protein solution upon homogenisation under the Tissuemizer. The addition of this non-ionic detergent was intended to disperse lipids

within solution that would subsequently settle in the pellet during high speed centrifugation. Alternative interfering substances, potentially including reducing agents such as uric acid, catchecholamines or iron may have interfered with the BCA protein assay (Pierce 2005a). A more direct, physical measurement of textural strength may have been more valuable. However, this would have only given a measure following thawing. Thus, an indication of the rate of change occurring throughout the duration of frozen storage, independent of the thaw process, would have been more difficult to obtain.

The Effect of Thawing Pre-rigor Chinook salmon White Muscle

The occurrence of metabolic activity during thawing of pre-rigor fish muscle is well documented (Ma, L.B. *et al.* 1992; Cappeln, G. & Jessen, F. 2001), but none the less remarkable. For enzymatic processes to occur in thawing tissue it demonstrates that metabolic enzymes are not irreversibly denatured by the freezing process. However, the rapid depletion of metabolic energy substrates concomitant with the rapidly increasing lactate concentrations in thawed tissue identifies that the cellular decompartmentalisation occurs involving rupture of the sarcolemma and lysosomes (Ma, L.B. *et al.* 1992; Nilsson, K. & Ekstrand, B. 1993), meaning freeze/thawed muscle no longer maintains controlled metabolic processes.

Teleost WM is functionally adapted to glycolytic metabolism, owing to low mitochondrial volumes, poor blood perfusion and the absence of myoglobin (Willmer, P. *et al.* 2005). Additionally, owing to the limited oxygen supply, anaerobic metabolic pathways predominate when WM is recruited in teleost (Wang, Y.X. *et al.* 1994; Willmer, P. *et al.* 2005). Thus, it is unsurprising that during thawing of pre-rigor WM tissue, metabolic activity is glycolytic in origin. It is unknown if any proportion of metabolic activity is aerobic as oxygen permeation to the mitochondria could well occur, although whether the mitochondria are still viable post-freezing is unknown. However, owing to the isolated nature of the tissue blocks and the rapid increases in tissue lactate observed, these ischemic metabolic processes are likely to be predominantly anaerobic.

Although not directly investigated within this thesis, depletion of high energy metabolites such as ATP and glycogen, upon thawing, are associated with the phenomenon of thaw rigor. Thaw rigor, appears to be caused by the rupture of the sarcolemma, resulting in the release of Ca^{2+} ions which activates high levels of Ca^{2+}/Mg^{2+} ATPase activity (Hultin, H.O. 1984; Gill, T. 2000). Consequently, a strong rigor of the muscle sets in, which has been cited as detrimental to the product quality (Jones, N.R. 1965).

The Effect of Rested Harvesting on Lipid Oxidation Processes in White Muscle

Within this thesis, processes of lipid oxidation in Chinook salmon WM were investigated. As mentioned previously, Chinook salmon WM is structured so to maintain a maximal density of large diameter muscle fibres, resulting in few blood capillaries, low mitochondrial volumes, and an absence of myoglobin (Love, R.M. 1980). These structural properties confer differences in the pro-oxidant activity of the tissue (such as the relative absence of heme proteins and associated metal species in the oxidised state) making WM comparatively resistant to lipid oxidation (when compared to the skin and red muscle) (Undeland, I. 2001).

The lipid composition of the WM varies with age and diet, but generally speaking phospholipids comprise 10-20% of the total WM lipid pool, while the triacylglycerols comprise 70-90% the total WM lipid fraction (Kiessling, A. et al. 2001). The phospholipids have a higher proportion of polyunsaturated fatty acids (≈50-60%), than the triacylglycerol fraction (10-20% polyunsaturated fatty acids) (Kiessling, A. *et al.* 2001). It is these phospholipids that are considered primarily to undergo oxidation during post mortem storage (Igene, J.O. *et al.* 1980; Erickson, M.C. 1997). The greater oxidation potential of the membrane associated phospholipids is linked to the surface area of the lipid exposed to the aqueous phase. Membrane associated phospholipids have 50-100 times greater surface area contact (on a weight basis) than the neutral lipids, which are typically found in oil droplets (Hultin, H.O. 1994).

Seen in this study, rested tissue showed a delayed development of lipid oxidation products (as measured by the TBARS assay) following transfer from frozen temperature storage to refrigerated temperatures (+4℃ and -1℃). No evidence of retarded lipid oxidation processes were identified within rested tissue during prolonged frozen storage at either -19℃ or -9℃. Additional findings identify that following one month storage at frozen temperatures (-19℃), the ability of rested tissue to retard lipid oxidation was markedly less than tissue frozen stored at -80℃, for up to three days, before transfer to -1℃. (Rested tissue stored at -20 then +4℃ demonstrated a significant increase two days after exercised tissue, whereas rested tissue stored at -80 then -1℃ showed a significant increase nine days after exercised tissue). It is unknown whether this is a temperature related effect, or an effect of duration of frozen storage prior to transfer to refrigerated temperatures. However these results could suggest that during extended periods of frozen storage, the capacity which rested tissue possesses to retard lipid oxidation products is diminished.

Mechanisms by which rested WM is able to retard lipid oxidation either relate to exercise and stress effects associated with the harvest procedure, or relate to the use of the rested harvest technique which utilises the anaesthetic AQUI-S™. Utilisation of AQUI-S™ is one of the more likely mechanisms retarding oxidation processes, owing to the antioxidant behaviour of isoeugenol. As discussed in Chapter 4, isoeugenol is a potent antioxidant within *in vitro* biological lipid systems (Rajakumar, D.V. & Rao, M.N.A. 1993; Priyadarsini, K.I. 1997; Ito, M. *et al.* 2005). It would then be expected that isoeugenol protects the polyunsaturated lipid components of WM tissue from oxidation, either by complexing free metal ions (Ito, M. *et al.* 2005) or by scavenging hydroxyl ion radicals (Rajakumar, D.V. & Rao, M.N.A. 1993).

Possible mechanisms in rested tissue that retard lipid oxidation processes may also relate to stress or exercise effects in tissue harvested by exercised or conventional means. Heme protein interactions involving the myoglobin, or the hemoglobin perfusing WM during exercised harvest protocols could potentially cause elevated levels of lipid oxidation within the WM of exercised tissue. Alternatively, fatty acid metabolic pathways or

hormonally induced lipolysis may have an effect on peri- or post mortem FFA hydrolysis and oxidation, leading to a higher propensity for exercised tissue to oxidize during storage at unfrozen temperatures.

Further study needs to be carried before the mechanism by which rested tissue retards lipid oxidation is identified. A simple experiment to determine whether isoeugenol retards lipid oxidation, or whether the rested physiological state is the causative agent, could be performed by a rested harvest protocol that utilizes a different anesthetic (such as MS222 or benzocaine). Comparison between treatments involving rested methods with this alternative anaesthetic, rested methods utilising AQUI-S™ and exercised methods would identify whether it is the rested state, or isoeugenol that creates altered lipid oxidation properties of the WM. However, as AQUI-S™ is the only registered food grade anaesthetic, practical applications of a variant anaesthetic are limited.

Within the thesis, the determination of isoeugenol residue within the WM tissue blocks was attempted prior to, and on completion of frozen storage. Determination of isoeugenol was based on detection by fluorometry. The wavelength at which isoeugenol fluoresces has been well characterised, however, the wavelengths at which breakdown products of isoeugenol fluoresce have not. Potentially, some breakdown products could fluoresce at the same wavelengths as isoeugenol, leading to difficulty in differentiating the breakdown products from the starting product – isoeugenol. More thorough investigation on the break down products of isoeugenol would need investigating using alternative analytical techniques such as GC-MS, or NMR.

Within Chapter 5 it was identified that rested tissue filleted six hours post harvest showed a significant increase in lipid oxidation products after 21 days storage at -1℃, when compared to tissue stored 24 hours post harvest, which only showed a significant increase after 24 days at -1℃. Overall, a near significant difference was observed between these two treatments, therefore the effect of time of filleting on lipid oxidation product development following frozen/refrigerated storage is worth investigating.

Issues Comparing Literature

Results from this investigation were compared to other results cited within the published literature. Comparison between different species (both salmonid and other teleosts) was required as no investigations into the storage properties of Chinook salmon were available. Difficulties presented in comparisons with other published literature include comparing fish with unknown life history traits, including diets, rationing and age. Also, in many cases, details of the experimental handling of the fish were often not disclosed, including the harvest procedures utilised, tissue preparation steps and pre-freezing storage routine.

Applications of this Research

As the harvest treatments adopted within this thesis are analogous to commercial practices, these results can be directly applied to fish harvested in commercial settings. Commercial scale implementation of rested harvest protocols can be easily achieved. Currently rested harvesting of Chinook salmon, utilising AQUI-S™, is being practiced within New Zealand and to a smaller extent, during the harvest of other salmon species throughout Chile and Australia.

Rested harvest protocols have been demonstrated to present a raw product with high concentrations of metabolic energy stores, which, when frozen prior to their depletion, can be maintained throughout the duration of storage at commercially relevant (<-19°C) temperatures. Upon thawing, rapid depletion of these high energy metabolites can potentially result in the generation of thaw rigor. Thaw rigor is considered detrimental to product quality, owing to increases in drip loss, textural changes and gaping (Jones, N.R. 1965; Jones, N.R. 1969). Avoidance of thaw rigor can be achieved by controlling temperatures upon thawing, whereby the storage temperature is raised to an intermediate temperature where depletion of high energy metabolites results in the development of rigor while the tissue is in the partially frozen state (Neilsen, M.K. & Neilsen, H.H. 2006).

As identified within this thesis, storage of Chinook salmon WM at -9℃ demonstrated such a depletion of ATP and glycogen, occurring after five and 10 weeks storage, respectively. Thus, increasing the storage temperature of frozen stored Chinook salmon to -9℃ will deplete metabolic energy stores, eliminating thaw rigor development. However, lipid oxidation products were also seen to increase after 10 weeks storage at -9℃. Consequently, storing of tissue for extended periods at intermediate temperatures as part of a two-step thawing regime would, although beneficial to thaw rigor avoidance, be detrimental to the quality of the resulting product (with reference to lipid oxidation). Thus, thaw rigor avoidance would probably be best achieved by a one step thawing regime. Transfer of Atlantic salmon fillets from -25℃ to +4℃, presented a suitable thawing regime where no evidence of thaw rigor was observed (Einen, O. *et al.* 2002). Similarly, ATP depletion has been observed in sea bream and carp muscle during thawing at temperatures below 0℃ (Ma, L.B. & Yamanaka, H. 1991).

Alternative means by which thaw rigor could be avoided in rested tissue could involve allowing stores of ATP to deplete during post mortem storage. In lowering the tissue concentrations of ATP and glycogen pre-freezing, lesser concentrations of ATP would be generated/metabolised upon thawing, hence lessening the extent of thaw rigor development. However, prolonged storage leading to the depletion of metabolic energy stores – allowing the whole fish to progress into rigor, would be detrimental owing to product quality losses associated with filleting whole-fish, in-rigor (e.g. fillet yield and tissue gaping) (Robb, D. 2002).

In Chapter 5, lipid oxidation processes in rested tissue exposed to a post mortem storage period were investigated. Results from this experiment were inconclusive, as the p-value identifying statistical differences between treatments was near the threshold of significance. Further retardation of lipid oxidation processes within rested tissue will result in a higher quality product, which will benefit the commercial operator. Whether rested tissue filleted towards the end of the pre-rigor period does possess quality benefits over rested tissue filleted immediately following slaughter requires further investigation. However, if delayed filleting does provide a quality benefit, this will allow post mortem

metabolism to deplete maintained metabolic substrates, combining to help reduce the development of thaw rigor, in frozen tissue.

With reference to rancidity development, rested harvest procedures have been found to retard lipid oxidation processes in thawed/chilled and unfrozen tissue (Chapters 4 & 5; Tuckey, 2008). However, no direct benefits were observed in frozen tissue as harvested by the rested (in comparison to exercised) tissue. Thus the benefits of rested harvesting (utilising AQUI-S™) to Chinook salmon producers are related to chilled product, and product removed from frozen storage. In relation to frozen product quality, retardation of lipid oxidation would most likely benefit the consumer, as following purchase (either in the frozen, or recently thawed state), fish harvested by rested means would showed delayed lipid oxidation (as has been shown by TBARS analysis).

It is expected, owing to similarities in biochemical composition, that rested harvesting would provide the same retardation of lipid oxidation processes in other salmonid species. Thus, quality improvement could be achieved in commercial species including Atlantic (*S. salar*), Coho (*O.kisutch*), rainbow/steelhead (*O. mykiss*) and Sockeye salmon (*O.nerka*). Lipid oxidation processes in other cold water fish such as the scombrids, which also possess high concentrations of polyunsaturated fatty acids, could potentially benefit from the rested harvesting protocols employed. Non-oily 'white fish', with lower concentrations of polyunsaturated fatty acids, which deposit predominantly in the liver (as opposed to muscle), are less prone to lipid oxidation (Hedges, N. & Neilsen, J. 2000). However, recent thinking proposes that the low level lipid hydrolysis and oxidation that does occur in 'white fish' contributes to losses in sensory quality, protein denaturation, textural changes and protein aggregation (Aubourg, S.P. et al. 2007). Thus, lipid oxidation processes may be a determining factor of the shelf life of 'white fish' products outside the frozen state. Potentially, quality could be improved by retarding lipid oxidation processes, delaying the interaction of lipid oxidation products with other functional components of the tissue and extending the quality life of the fish. Experimental evidence is required to test these assumptions.

This was a brief investigation into some deteriorative processes that occur during frozen storage and upon thawing of Chinook salmon WM. Application of this data set to predict shelf life would not be prudent, owing to the small size of tissue blocks committed to frozen storage. Blocks of WM utilised possessed a much larger surface to volume ratio than whole fillets of tissue, as typically presented in wholesale and retail settings. Additionally, TBARS as a measure of lipid oxidation, does not always correlate well with sensory determinations of rancidity (Hoyland, D.V. & Taylor, A.J. 1991; Erickson, M.C. 1993; Huss, H.H. 1995). Furthermore, in a study on cultured bass species (*Morone spp.*) TBARS values were found to be most suitable for describing the early stages of lipid oxidation, whereas other measures of lipid oxidation, including conjugated dienes, fluorescent product development and headspace volatiles, were more suited to measuring later stages of lipid oxidation (Erickson, M.C. 1993). Collectively the data sets presented do not provide a thorough determination of rancidity processes occurring within frozen and thawed Chinook salmon WM. In order to determine the shelf life of product, further analysis needs to be carried out, this would include volatile and biogenic amine analysis, sensory taste tests, volatile organic and sulfur compound analysis and adenosine catabolites analysis (Dalgaard, P. 2000).

Future Directions of Study

Further Confirmation of Results

One of the main unanswered questions of this thesis is whether post mortem storage of rested WM, prior to filleting, confers an enhanced ability to retard lipid oxidation processes, when compared to rested tissue filleted immediately following harvest. Results from this thesis are not clear cut and further experimentation will hopefully identify whether the time of filleting has a significant effect on oxidative processes during storage. The potential for viable WM to undergo oxidative stress (ROS generation and, or, antioxidant depletion) upon excision could potentially have a significant effect on the way that high quality, rested fish are processed. However, quality gains attained by delayed filleting could potentially incur additional costs to the producer.

Further investigation of other lipid oxidation processes that occur during frozen, then chilled storage will help to further elucidate the benefits that rested harvesting provides to retard lipid oxidation processes. Ascertainment of later lipid oxidation products, including the conjugated dienes, volatile hydrocarbons and tertiary lipid oxidation products will assist in this investigation.

Results from this thesis provide further support for the incorporation of rested harvested protocols into commercial harvests of salmonid species. Application of humane harvest practices are further encouraged by the quality gains that are associated with the harvest method. Whether lipid oxidation processes and rancidity are the critical factors that limit frozen then chilled product needs to be determined. However, even if off flavour or odour development owing to lipid oxidation processes was not to be considered the first conspicuous process that renders a product undesirable, it would provide a further avenue that could be addressed to improve product quality.

Further Understanding of the Current Results

Whether the ability of rested tissue to retard lipid oxidation processes is associated with the uptake of isoeugenol, or is a stress/exercise associated effect is yet to be determined. As mentioned previously, comparison of rested tissue harvested using an alternative anaesthetic, to rested tissue harvested with AQUI-S™ should provide support for either hypothesis.

In understanding whether exercise does have an effect on post harvest lipid oxidation processes, the role of peri-mortem FFA lypolysis and mobilisation as well as hemoglobin perfusion into WM could also be investigated.

Rested tissue has been demonstrated to possess enhanced textural properties, when compared to tissue harvested by exercised means (Kiessling, A. *et al.* 2004). The mechanism by which this occurs has not yet been identified, as it does not appear to be related to changes in the solubility of myofibrillar proteins (Chapters 3 and 4).

Within this thesis, WM stored at +4°C following four weeks frozen storage showed a lesser ability to retard lipid oxidation than WM stored at -1°C following three days storage at -80°C. Further experiments to identify whether increasing periods of frozen storage limit the ability of rested tissue to retard lipid oxidation processes, or whether -1°C provides a more beneficial storage temperature (c.f. +4°C) would provide valuable information. Additional investigation into why rested harvesting does not retard lipid oxidation processes in WM stored at frozen temperatures would also be beneficial, and might help to explain potential differences in the time/temperature relationship suggested above. This could probably be progressed and supported by investigating *in vitro* isoeugenol-lipid chemistry at frozen temperatures.

Further Investigations of Frozen Storage Quality

Other possible avenues of research to arise from this research include the tying of glass-state phase transitions to stability of cellular metabolites. DSC of tuna and cod identify the existence of phase transition zones at -11 to -13°C and -18 to -21°C (Jensen, K.N. *et al.* 2003). These temperatures correlate with observations within these results, where storage at -9°C (a temperature above the phase transitions observed in cod and tuna) was associated with a gradual decrease of tissue ATP and glycogen stores. Whereas, during storage at -19°C (within the cited phase transition zone) no depletion of cellular metabolites was observed. Calorimetry of Chinook salmon would identify whether the same phase transition exists, and experiments utilising storage temperatures above and below each phase transition would be informative. DSC could also be incorporated into thaw studies where heat output during the thaw, potentially identifying the precise temperature at which cellular activity occurs in pre-rigor frozen muscle, could be defined.

Post mortem metabolism in tissue removed from frozen storage is significantly different from the post mortem metabolism of freshly harvested tissue. High concentrations of ATP and glycogen have been demonstrated to fuel post mortem metabolism, and extend the pre-rigor period in 'chilled' tissue (Jerrett, A.R. *et al.* 2000). However, within frozen tissue, high concentrations of metabolic stores are depleted immediately upon thawing (Chapters 4 and 5). To be able to control the thaw metabolism of pre-rigor frozen tissue

would present numerous advantages, associated with elimination of thaw-rigor, and quality enhancement. The physiology of freeze tolerant frogs allows the protection of cellular organelles and control of metabolic processes, enabling their survival through both freezing and thawing events (Storey, K.B. & Storey, J.M. 2004). To replicate this form of cryo-protection, through the addition of cryo-protectants *in vivo*, could potentially provide a gateway to further quality improvements of frozen tissue. Possible cryo-protectants would have to have a low molecular mass and be non-toxic allowing uptake to the interstitial fluid. Additionally, the cryo-protectant would have to be fit for human consumption. *In vivo* addition could potentially occur across the gills in the anaesthetised animal, or by submersion of fillets in a cryo-protectant containing solution. Likely cryo-protecting candidates include the low molecular weight carbohydrates.

Closing Comments

Work undertaken within this thesis has provided new information on the storage properties of Chinook salmon, the sole salmonid species cultured for food production in New Zealand. Findings that detail the frozen storage and thaw properties of tissue harvested by exercised and rested means, provides information which commercial processors and retailers could implement in their efforts to improve product quality.

Further information supporting the incorporation of rested harvesting protocols, utilising the aquatic anaesthetic AQUI-S™, has been provided. Providing tissue with the ability to retard lipid oxidation processes in chilled tissue, once removed from frozen storage, provides significant product quality benefits within oily fish species. Additionally, this benefit is achieved with no further processing steps, following the harvest event. Furthermore, in implementing rested harvest protocols, not only are product quality benefits being obtained, a humane and ethical harvest is performed.

Acknowledgments

The Foundation of Research Science and Technology, for granting me the TIF fellowship (Contract# AQU0502) and AQUI-S NZ Ltd for providing me with this opportunity.

Malcolm Forster, for his assistance and academic input as supervisor. For his mentoring and support within aspects of research, my future direction and life outside the University. You have helped to make this MSc program a thoroughly enjoyable and rewarding experience.

Jan Holland and Don Bell of AQUI-S® NZ Ltd. Jan for her help in formulating this research project, and her depth and breadth of knowledge on, seemingly, all things harvesting and aquaculture. Don for his support of this research topic and the provision of AQUI-S®.

Alistair Jerrett, Suzie Black and the team at Crop and Food, Nelson. Many thanks for their support of this project. For providing, and allowing my intrusion during, the period of field work conducted in Marlborough Sounds. Further thanks for their assistance in formulating the research plan, and the support with analytical techniques and fish handling procedures.

Steven Gieseg and other members of the FRB Lab, for allowing the encroachment into, and the use of their laboratory. Special thanks to Nick Tuckey for his patience and support during my learning of the HPLC analysis techniques, as well as the numerous discussions on work surrounding this thesis.

Mike Field-Dodgson and the team at Isaac Salmon, for the provision of fish and use of the hatchery facilities. Without which, would make our research near impossible.

The technicians, Gavin Robinson, Linda Morris and Claire Galilee for the running around collecting all matter of materials involved in this project.

Other students of the Physiology Lab. Len for his assistance and discussions. Gareth for providing the extra pair of hands, which were often required.

Many thanks to Kerryn, my new wife. For moving to Christchurch and putting off her own study plans to support me over this period of study.

My Family, for their encouragement and support since leaving home, all those years ago.

The Crosbie Family, for taking me in during my brief period of homelessness in Christchurch.

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